BIOCHIMICA
et
BIOPHYSICA ACTA

INTERNATIONAL JOURNAL OF BIOCHEMISTRY AND BIOPHYSICS
REVUE INTERNATIONALE DE BIOCHIMIE ET BIOPHYSIQUE
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VOL. 40 1960

ELSEVIER PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands by

DRUKKERIJ MEIJER WORMERVEER AND AMSTERDAM

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ERRATA

BIOCHIMICA ET BIOPHYSICA ACTA, VOL. **40** (1960)

Page 137:

Line 3: Change "during" into "after".

Legend Fig. 3: Add "The fine curve indicates hydrolysis of ITP.".

Line 9: Between "phase" and "ATPase" insert "of".

Page 138:

Line 6: Delete "B".

ERRATA

BIOCHIMICA ET BIOPHYSICA ACTA, VOL. 46 (1961)

Page [illegible]

Line 3: Change "during" into "after".

Legend Fig. 4 A and [illegible]

Line 9: Between "[illegible]" and "ATPase" insert [illegible]

Page 128

Line 8: Change [illegible]

FLAVIN NUCLEOTIDES AND LIGHT-INDUCED PHOSPHORYLATION IN CELL-FREE EXTRACTS OF *RHODOSPIRILLUM RUBRUM*

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(Received August 17th, 1959)

SUMMARY

Light-induced phosphorylation (LIP) in extracts of the photosynthetic bacterium *Rhodospirillum rubrum* can be stimulated by addition of FAD, but not by addition of FMN. Atebrin, a flavin antagonist, strongly inhibits LIP. This inhibition can be completely overcome with FAD but only partially with FMN.

The inhibition of LIP by atebrin is equally strong in the presence of phenazine methosulfate, which stimulates LIP, as in its absence. Thus, in contrast to the case when antimycin A or 2-*n*-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) are used as inhibitors, phenazine methosulfate does not provide a "by-pass" around the point of action of atebrin.

Antimycin A and HOQNO inhibit LIP almost completely both in the presence and in the absence of added FAD. This indicates that any electrons passing through FAD also pass through the site which is inhibited by antimycin A and HOQNO.

It is suggested that FAD is an electron transport carrier in LIP in cell-free extracts of *Rhodospirillum rubrum*.

INTRODUCTION

The light-induced phosphorylation (LIP) reactions in bacterial[1] and in plant[2] systems are assumed to be linked to electron transport. The main experimental reasons for this assumption are: (1) addition of various known electron carriers stimulate LIP[2-4], (2) typical respiratory chain inhibitors inhibit LIP[5-7] and, in extracts of a bacterium, the facultative phototroph *Rhodospirillum rubrum*, (3) results from spectrophotometric studies indicate the participation of cytochromes in LIP[8].

Knowledge about the details of electron transport in bacterial LIP is very limited. FRENKEL's[1] original scheme gives the sequence (H) → C → (OH), where C is the oxidized form of hypothetical electron carriers, which are coupled to LIP. (H) and (OH) are the symbols for the reduced and the oxidized product, respectively, of the bacterial equivalent to the Hill reaction. SMITH AND M. BALTSCHEFFSKY[5] have shown that HOQNO in low concentrations strongly inhibits LIP in *Rhodospirillum rubrum*, and GELLER[6] has obtained the same effect with antimycin A. As SMITH AND M.

Abbreviations: P, orthophosphate; ATP, adenosine triphosphate; FAD, flavinadenine dinucleotide; FMN, flavin mononucleotide; PMS, phenazine methosulfate; HOQNO, 2-*n*-heptyl-4-hydroxyquinoline-N-oxide; *M*, moles per liter; % P_{org}, percentage orthophosphate esterified.

BALTSCHEFFSKY[8] have demonstrated, C in FRENKEL's scheme appears to include a *b*-type cytochrome and cytochrome c_2. In the presence of HOQNO they observed that the *b*-type cytochrome became reduced and cytochrome c_2 oxidized, indicating that the inhibitor blocked electron transport from the *b*-type cytochrome to cytochrome c_2. CHANCE[9] has shown that both antimycin A and HOQNO block the respiration in animal mitochondria between cytochrome *b* and cytochrome c_1, and the assumption has been made[7] that the point of action of the two inhibitors is the same in the two electron transport systems.

No data have been found in the literature about a possible role of flavin compounds in bacterial LIP. The present paper is centered on the role of flavin nucleotides in LIP in cell-free extracts of *Rhodospirillum rubrum*. FAD but not FMN stimulates this LIP. Low concentrations of atebrin, a flavin inhibitor, inhibit LIP. FAD and to a lesser degree FMN counteract this inhibition. The above findings support the conclusion that flavin is involved in the electron transport of LIP in extracts of *R. rubrum*, and that the flavin in question is FAD.

MATERIALS AND METHODS

Materials

ATP, FAD and FMN were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. FAD was of the 80–90 % type. HOQNO was an appreciated gift from Dr. J. W. CORNFORTH, National Institute for Medical Research, London, England.

Culture of bacteria and preparation of cell-free extracts

Rhodospirillum rubrum, van Niel Strain S1, were obtained from Dr. J. M. OLSON at Brandeis University, Waltham, Mass., U.S.A. The bacteria were grown in completely filled glass-stoppered bottles under illumination from incandescent, 150-W Philips lamps, at 30°, in essentially the medium described by GEST, KAMEN AND BREGOFF[10]. The only differences were that the concentrations in mg/l of Difco yeast extract and of biotin were 500 and 0.010 instead of 250 and 0.005 respectively, in agreement with the advice of Dr. M. D. KAMEN at Brandeis University, Waltham, Mass., U.S.A. The bacteria were harvested in the resting phase.

To obtain the extracts the bacteria were first centrifuged down in an International Refrigerated Centrifuge, Model PR 1 (Head No. 840), for 10 min at 4,100 × *g**. The pellet was washed once with distilled water and recentrifuged as above. These operations were repeated once with 0.02 *M* glycylglycine pH 7.4 instead of distilled water. The pellet so obtained was weighed and ground in a porcelain mortar with a 2- to 3-fold excess of alumina (Alcoa A-301). After 3 min grinding at 0° the material was taken up in 0.2 *M* glycylglycine buffer (about 3 ml buffer per g pellet) and centrifuged in the Spinco refrigerated centrifuge, for 10 min at 10,000 × *g*, in rotor 40, which also was used for the subsequent centrifugations. The supernatant from this centrifugation** was recentrifuged for 60 min at 25,000 × *g*. The sediment was then washed once in 0.2 *M* glycylglycine and recentrifuged for 60 min at 25,000 × *g*. This sediment was taken up in a small volume of 0.2 *M* glycylglycine and stored in the dark at 0° as the "chromatophores" containing fraction.

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* The values given are for the bottom of the tube with the International Refrigerated Centrifuge and the middle of the tube with the Spinco Refrigerated Centrifuge.

** This material had been used in the work described in a preliminary, short communication on LIP in *R. rubrum*[7]. In contrast to the other inhibitors, atebrin did not in the long run give reproducible effects with this "crude extract". Thus the value for atebrin inhibition in the above-mentioned communication is not valid.

The supernatant from the first centrifugation at 25,000 × *g* was centrifuged for 90 min at 100,000 × *g*. The sediment from this centrifugation was washed once in 0.2 *M* glycylglycine and recentrifuged for 90 min at 100,000 × *g*. This sediment was taken up in a small volume of 0.2 *M* glycylglycine and stored in the dark at 0° as the "chromatophore fragments" containing fraction. (The names of the fractions should be regarded as functional only; they do not imply any degree of homogeneity.)

Measurement of LIP

The amount of phosphate esterified in the LIP experiments was measured by the ^{32}P method recommended by LINDBERG AND ERNSTER[11]. FRENKEL[1] has shown that the ATP formed in LIP is equivalent to the amount of inorganic phosphate that has disappeared. This was confirmed by paper chromatography* employing the method of KREBS AND HEMS[12].

The efficiency of LIP was measured and related to the concentration of chlorophyll according to the method used by FRENKEL[1] as µmoles of phosphate esterified per hour per unit absorbancy at 800 mµ ("OD_{800}").

The medium employed for the LIP measurements contained, unless otherwise noted, 1.5 ml 0.2 *M* glycylglycine pH 7.4, 8 µmoles $K_2H^{32}PO_4$, 10 µmoles ATP, 30 µmoles $MgCl_2$, 1 µmole sodium succinate, 60 µmoles glucose and an excess of yeast hexokinase**. The volume during the experiment was 3 ml. The reaction was stopped with 1 ml 2 *M* perchloric acid.

The "chromatophore fragments" fraction was used in all the experiments reported here. The temperature of the water-bath which contained the test-tubes with the reaction mixture was 30°. A saturating light-intensity was always used. Since the efficiency of LIP was roughly the same under aerobic as under anaerobic conditions[14], aerobic conditions were chosen. In order to "avoid complications due to light-induced reactions involving molecular oxygen" FRENKEL[1] used anaerobiosis for his investigations. The possibility that O_2 might serve as an alternative to (OH) as the oxidizing agent in LIP cannot be excluded when aerobic conditions are used.

In all experiments two controls were run. In one of them the reaction was stopped at zero time. The other one was a dark control, which was wrapped in aluminium foil and run parallel with the other tubes. Their % P_{org} was often negligible and seldom exceeded 1.5.

RESULTS

Fig. 1 shows that FAD stimulates LIP in washed chromatophore fragments from *R. rubrum*, whereas FMN does not. Here, as in most of the experiments, the stimulation reaches a value of between 50 and 100%, but occasionally less than 50 or more than 100% stimulation has been obtained. FMN has an inhibitory effect on the LIP. The effect is small and usually more pronounced at lower concentrations of FMN than at higher, as can be seen from Fig. 1.

---

[*] Dr. T. E. CONOVER kindly guided and assisted in these experiments.

[**] The hexokinase was obtained from Dr. L. ERNSTER and Dr. H. LÖW. It had been prepared and stored as described in ref.[13].

The inhibition of LIP by atebrin is demonstrated in Fig. 2. At a concentration of $4 \cdot 10^{-5}$ *M* atebrin 50% inhibition is obtained. This value is not entirely constant from preparation to preparation. The highest concentration of atebrin required for 50% inhibition has been $1.2 \cdot 10^{-4}$ *M* and the lowest $2 \cdot 10^{-5}$ *M*.

The effect of FAD and FMN on atebrin-inhibited LIP is shown in Fig. 3. Both of the flavin nucleotides can restore LIP, but FAD is more efficient than FMN. Only with FAD has a complete restoration been obtained. For example, in an experiment

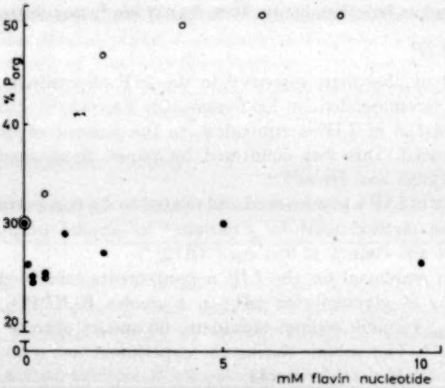


Fig. 1. Effects of FAD and FMN on LIP. In each tube "OD$_{800}$" = 0.21. 30 min experiment. ○ = FAD, ● and ◐ = FMN. Two separate experiments with FMN are included in order to show the limit of inhibition obtained at low concentrations. In the ◐-series the % P$_{org}$ was 34 without added FMN. For the sake of clarity this was reduced to 30 in the figure and the other values were multiplied by the same factor.

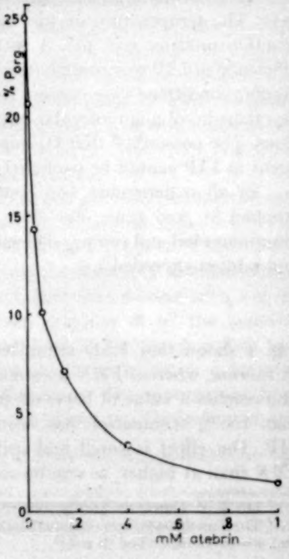


Fig. 2. Inhibition of LIP with atebrin. In each tube "OD$_{800}$" = 0.19. 30 min experiment.

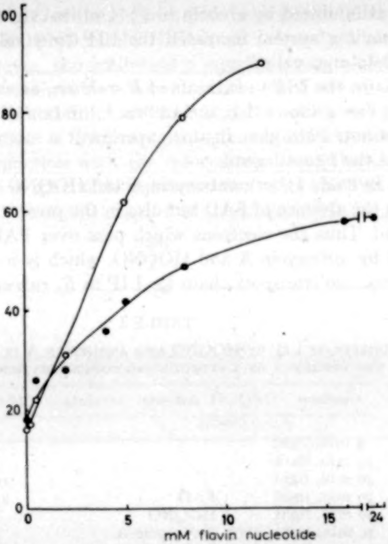


Fig. 3. Restoration of LIP by FAD and FMN in an atebrin-inhibited system. 30 min experiment. The value obtained with no atebrin added corresponds to 100% initial activity. In all other tubes the atebrin concentration was $2 \cdot 10^{-4}$ *M*. ○ = FAD, ● = FMN.

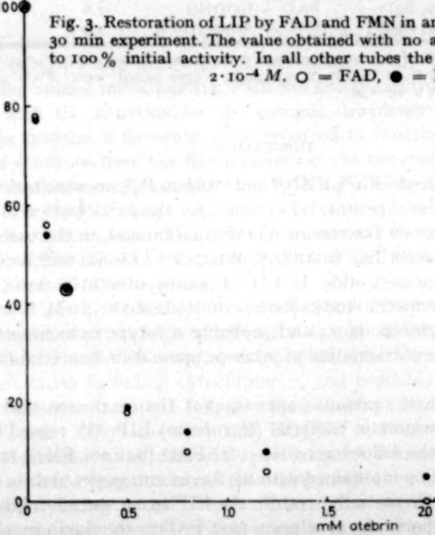


Fig. 4. Inhibition of LIP by atebrin in the absence and presence of PMS. 6 min experiment. ○ = without PMS (100% initial activity = 13 μmoles P esterified per hour per "OD_{800}"). ● = with PMS (100% initial activity = 54 μmoles P esterified per hour per "OD_{800}"). The final concentration of PMS, where added: 0.01%.

in which LIP was inhibited by atebrin to 63 % of its original value, 12 mM FAD in the atebrin-containing system increased the LIP to a value that was 25 % above the original, uninhibited value.

PMS stimulates the LIP in extracts of *R. rubrum*, as was first shown by GELLER AND GREGORY[15]. Fig. 4 shows that the atebrin inhibition of LIP is the same whether PMS is added or not. PMS gave in this experiment a more than 4-fold stimulation, as is indicated in the figure legend.

As is shown in Table I, both antimycin A and HOQNO almost completely inhibit LIP, not only in the absence of FAD but also in the presence of stimulating amounts of this compound. Thus the electrons which pass over FAD also pass over the site that is inhibited by antimycin A and HOQNO, which is to be expected if FAD is a member of the electron transport chain for LIP in *R. rubrum*.

TABLE I

THE INHIBITION OF LIP BY HOQNO AND ANTIMYCIN A IN THE ABSENCE AND THE PRESENCE OF A STIMULATING CONCENTRATION OF FAD

Conditions	*Additions*	*LIP (% P_{org})*
0 min, light		1.2
30 min, dark		1.1
30 min, light		16
30 min, light	FAD	23
30 min, light	HOQNO	1.5
30 min, light	Antimycin A	2.6
30 min, light	FAD + HOQNO	2.6
30 min, light	FAD + Antimycin A	1.9

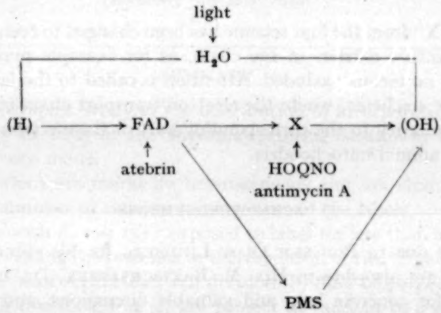
The final concentrations of the following compounds, where added, were: FAD 3 mM, HOQNO $2 \cdot 10^{-6}$ M and antimycin A $2 \cdot 10^{-6}$ M.

DISCUSSION

Several compounds, such as TPN[16], FMN[17] and vitamin K_3[2], are assumed to be electron carriers in plant LIP. The experimental evidence for the participation of these agents is their stimulating effect on the rate of ATP-formation and, in the case of TPN, the phosphorylation which according to ARNON, WHATLEY AND ALLEN[18] is coupled to the reduction of the pyridine nucleotide. In LIP of photosynthetic bacteria (*R. rubrum*), results from spectrophotometric studies have permitted SMITH AND M. BALTSCHEFFSKY[8] to suggest a role for cytochrome c_2 and probably a *b*-type cytochrome as electron carriers. No evidence for participation of other compounds in bacterial LIP has so far been reported.

The results given in the present paper support the conclusion that flavin takes part in the electron transport in bacterial (*R. rubrum*) LIP. We regard the evidence presented as strong for the following reasons: (1) FAD (but not FMN) stimulates the LIP, (2) powerful inhibition is obtained with the flavin-antagonist atebrin and (3) FAD can completely (and FMN partially) restore the LIP in an atebrin-inhibited system. The above evidence supports the conclusion that FAD is the flavin involved. In this connection it might be mentioned that PEEL[19] has determined the content of flavins of light-grown *R. rubrum*, and found FAD, FMN and riboflavin, FAD being present in the highest concentration.

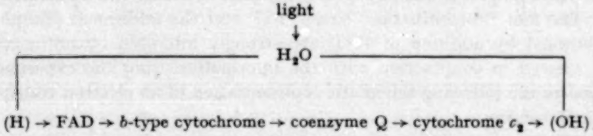
PMS provides a short-cut for the electron flow from (H) to (OH) around the site where antimycin A and HOQNO inhibit LIP in *R. rubrum*[7]. When the system is inhibited by atebrin, however, the inhibition is equally strong whether or not PMS is present. This shows that atebrin does not inhibit the same site as antimycin A and HOQNO. The fact that both the "basal" LIP and the additional phosphorylation that is obtained by addition of FAD are strongly inhibited by antimycin A and HOQNO, viewed in conjunction with the information from the experiments with PMS, supports the following schematic representation of an electron transport chain for LIP in *R. rubrum*:



In this scheme, flavin has been put on the reducing side of the site "X", which is inhibited by HOQNO and antimycin A, in analogy with the known sequence in phosphorylating animal mitochondria. PMS will accept electrons from FAD, and this is in analogy with the situation for the succinic dehydrogenase of KEARNEY AND SINGER[20]. This enzyme, a flavoprotein, is reported to function optimally with PMS as acceptor of electrons from the flavin moiety of the enzyme[21, 22].

The fact that atebrin inhibits LIP equally in the presence of PMS as in its absence seems to indicate that the component which atebrin acts upon takes part in the rate-limiting reaction in both cases. This is in agreement with the given scheme, where the rate-limiting reaction would be between FAD and "X" in the absence of PMS and between FAD and PMS in its presence.

The above scheme, which accounts only for results from inhibitor studies, can be combined with the one that is given by SMITH AND M. BALTSCHEFFSKY[8] on the basis of results obtained with difference spectrophotometry. These authors suggest that a "cytochrome chain including cytochrome c_2 and probably a *b*-type cytochrome" mediates the electron transport between the reductant and the oxidant from the photolysis of water. They also show that HOQNO inhibits electron transport from the *b*-type cytochrome to cytochrome c_2. It may be assumed that flavin is on the reducing side of the *b*-type cytochrome, in analogy with the sequence in the respiratory chain of phosphorylating animal mitochondria, and in microsomes. In the laboratory of GREEN[23] strong experimental evidence has been obtained that antimycin A acts on a rather ubiquitously occurring quinone, coenzyme Q, to which a role is ascribed in mitochondrial electron transport between cytochrome *b* and cytochrome *c*[24]. CRANE[25] has recently reported that coenzyme Q is present in large amounts in *R. rubrum*. It seems reasonable to assume that antimycin A acts on this compound also in the LIP of this bacterium. The following tentative formulation for the electron transport chain in LIP of *R. rubrum* is given on the basis of the above information:


light
H_2O
(H) → FAD → *b*-type cytochrome → coenzyme Q → cytochrome c_2 → (OH)

In this scheme, "X" from the first scheme has been changed to coenzyme Q. A possible role for other electron carriers in the chain, as for example pyridine nucleotide or vitamin K, is by no means excluded. Attention is called to the fact that it has been possible to derive a scheme, where the electron transport chain in LIP of *R. rubrum* shows a great similarity to the corresponding part of the electron transport chain of phosphorylating animal mitochondria.

ACKNOWLEDGEMENTS

Many thanks are due to Professor Olov Lindberg for his stimulating advice and support. Thanks are also due to Mrs. M. Baltscheffsky, Dr. Lars Ernster and Dr. Hans Löw for concrete help and valuable discussions, and to Mrs. Ingalill Lindgren for excellent technical assistance.

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THE ORGANIZATION AND REPLICATION OF DEOXYRIBONUCLEIC ACID IN THYMINE-DEFICIENT STRAINS OF *ESCHERICHIA COLI*

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(Received July 21st, 1959)

SUMMARY

1. An autoradiographic study of the distribution of incorporated [^{3}H]thymidine among the progeny of cells of the thymine-requiring strains of *Escherichia coli*, 15T$^-$ and B-3, has been made.

2. The distributions are markedly heterogeneous and are characterized by the presence of a small number of cells containing most of the label.

3. Experiments with *E. coli* 15T$^-$ exposed to label for less than one division time are interpreted as revealing the presence in this organism of two large DNA-containing structures which (a) must separate at cell division (b) may be perpetuated intact for many successive cell divisions, but (c) are subject at random to a finite probability of fragmentation during cell division. The possibility that these structures may be the two subunits of one larger structure is discussed.

INTRODUCTION

In a previous communication[1], it was reported that the distribution of incorporated [^{3}H]thymidine among the progeny of cells of a thymine-requiring mutant of *Escherichia coli* (strain 15T$^-$)[2], is very heterogeneous. We wish to discuss these results more fully in the context of some new findings.

MATERIALS AND METHODS

Bacteria

The bacterial strains used were the thymine-requiring organisms, *Escherichia coli* 15T$^-$, and *Escherichia coli* B-3, the latter initially isolated by Dr. S. BRENNER and obtained through the kindness of Dr. N. SYMONDS.

Media

The liquid culture medium was a phosphate-buffered, mineral salts medium known as M-9[3], supplemented with 4 μg/ml of either non-radioactive or [^{3}H]thymidine (Schwarz Laboratories Inc., Mt. Vernon, N. Y.). Specific activities ranging from 360 mC/m*M* to 3.0 C/m*M* were used during the course of the work.

Abbreviation: DNA, deoxyribonucleic acid.

Agar medium consisted of 0.8 % nutrient broth and 1 % Bacto-agar sometimes supplemented with varying amounts (40 or 400 μg/ml) of non-radioactive thymidine. In a few experiments, M-9 medium containing 1 % agar and varying quantities of thymidine was used.

Washing medium consisted of M-9 without glucose, supplemented with varying concentrations (4 or 400 μg/ml) of thymidine.

Labeling of cells

Radioactive liquid medium was inoculated by simple dilution of unlabeled log-phase cells growing at concentrations between 1 to $5 \cdot 10^8$/ml. The dilution factor was at least 1 to 10 in order not to alter appreciably the specific activity. No detectable change in the growth rate of the cells following inoculation occurred with this procedure. Cell counts were performed with a Petroff-Hausser counter. The extent of growth on label was an experimental variable to be discussed with the results. Radioactive cells were washed 2 or 3 times and resuspended in chilled washing medium.

Labeling was performed at 30° for *E. coli* $15T^-$ and 37° for *E. coli* B-3.

Growth on agar and micromanipulation

Small droplets of labeled cells were transferred to thin layers of non-radioactive agar medium deposited on 22 × 60 mm No. 2 coverslips. After cleaning, these coverslips had been pretreated for subsequent autoradiography by dipping into a solution of 0.1 % Knox deionized gelatin and 0.01 % chrome alum. Such coverslips are sufficiently thick (0.18–0.25 mm) to permit handling without significant breakage in autoradiography. The coverslips bearing the agar were placed on moist chambers. Two types of experiments were performed. On some coverslips, individual cells on the agar were permitted to grow to small colonies of varying size. On other coverslips, individual cells were isolated initially by micromanipulation and complete progeny lines isolated from these for several generations. The micromanipulation was performed by dragging cells on the agar surface with microneedles using a Chamber's micromanipulator. The position of individual cells was recorded by means of a graduated microscope stage with suitable reference markings on the coverslips. Usually, the incubation on agar was performed at room temperature (22 ± 2°) although some colonies were grown at 37°.

Autoradiography

The coverslips were handled for stripping-film autoradiography as previously described[4] except that the concentration of parlodian solution was reduced to 0.5 % to limit the absorption of β-rays by this layer. Cells isolated by micromanipulation were located by coordinate transformation on the graduated microscope stage of a Zeiss W-2 phase-microscope. Grain-counting was performed in brightfield under oil at 2000 × magnification. Recorded grain-counts were corrected for the trackiness that can be seen in such tritium autographs. It is not possible to count accurately more than a few disintegrations emanating from the same small locale (approx. 1 μ^2) because of the short-range of the low-energy tritium electrons. For grain-counts in excess of about 6 from such "point" sources of activity, the count will, in general, underestimate the activity compared with a distributed source containing the same amount of radioactivity.

RESULTS

When *E. coli* 15T$^-$ or *E. coli* B-3 is grown for many generations in radioactive medium to label fully the DNA, the subsequent distribution of this incorporated label to the progeny of individual cells is illustrated by the colony shown in Fig. 1. More of the detail of such clonal distributions can be seen in Fig. 2, which shows the grain-count data of typical progeny-lines from micromanipulation experiments. Notable and typical features of the distributions which have in part been summarized previously[1] are: (a) All-nothing distribution of label at division occurs frequently and can appear as early as the 3rd generation (8 progeny cells). (b) Such all-nothing distribution to daughters takes place successively in some sublines for several generations. In almost every progeny-line one can find at least one example of such perpetuated all-nothing

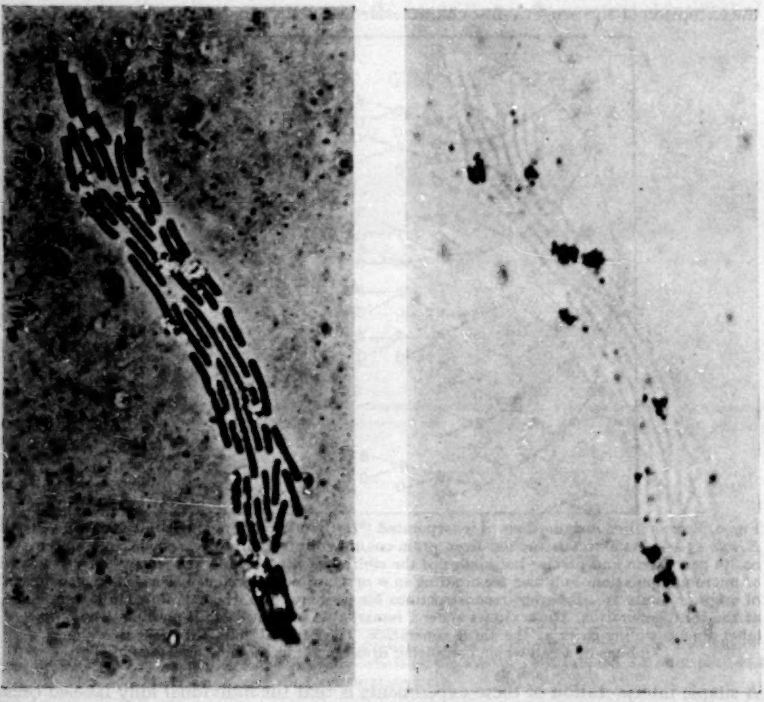

Fig. 1. Autoradiograph of colony of *E. coli* 15T$^-$ grown from a single cell fully-labeled with [^{3}H]-thymidine. Note the clustering of grains over relatively few cells, indicating the heterogeneous distribution of the original DNA among the progeny. (a) Phase to show cells clearly. (b) Brightfield to show photographic grains clearly.

divisions. In some cases, these have been seen to occur for as many as 7 successive generations. This number might be even higher if progeny-lines were isolated for more generations. (c) In other sublines, in addition to all-nothing splits, one finds examples of partition of the label to both daughters. This partition leads to the appearance of different numbers of hot-spots in different clones in generations after the third. (d) Search for colonies or progeny-lines with all-nothing distribution at the second generation produced a few suggestive examples but only at low frequency. (e) Partition of the label at the first division was always approximately equal (with the exception of one progeny-line characterized by many morphological abnormalities).

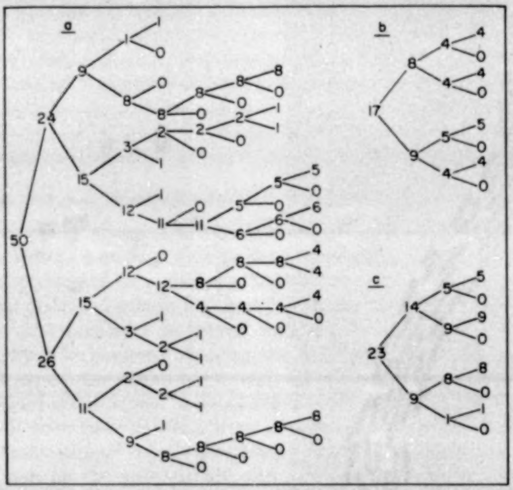

Fig. 2. Progeny-line distributions of incorporated $[^{3}H]$thymidine from single fully-labeled cells of *E. coli* $15T^-$. *a* is a reconstruction from grain-counts collected from individual cells of the 7th or 8th generation and precise knowledge of the sibling relationships in the family tree by means of micromanipulation. Sublines terminating in 0 or 1 are not extended beyond their generation of origin. *b* and *c* are analogous reconstructions for progeny-lines whose grain-counts were made at the third generation. These clones show a remarkable incidence of all-nothing distribution of label among sibling-pairs at the third generation. The original cells for these two clones were siblings of a cell which completed division at the moment of isolation.

A simple interpretation of these experiments is that the individual fully labeled cells have a minimum of four DNA containing structures which must separate from each other at division and which are subject to a certain probability of fragmentation during cell division.

In order to explore further the significance of this number four, experiments were performed in which cells of *E. coli* $15T^-$ were grown on label for one-half a generation time or less, preceding study of the distribution of label. Before presenting these results, it will be useful to discuss briefly the possible relations of DNA synthesis to the morphological features of the division cycle in *E. coli* $15T^-$.

In Fig. 3a, we have represented schematically the growth of *E. coli* $15T^-$ on M-9 medium. We have not specifically employed stains for nuclear bodies. However, cells observed in phase-contrast or in increased-contrast brightfield after autoradiography appear to contain relatively transparent objects. From the shape, location, and number of these objects in relation to cell elongation in the division cycle, it seems reasonable that they can be identified as the so-called nuclear-bodies described by others[5]. Thus, we have drawn a cell which has just divided as containing one such "nuclear body".

It would be convenient for these kinds of experiments, if DNA synthesis were confined to a limited portion of the division cycle. This question has been examined in our laboratory[6], and by others[7] with the result that such a restricted interval of synthesis does not seem to occur during logarithmic growth. This being the case, one cannot make the assumption that the completion of synthesis of any fundamental DNA-containing structures within a bacterium is necessarily coincident in time with the separation of two daughter cells. Rather, it seems more general to indicate the DNA-content of cells in the manner shown in Fig. 3b. Here, for sake of simplicity, we have made the assumption that there is one fundamental DNA-containing structure with a definite quantity of DNA, represented by the number 1. A cell which has just divided is depicted as having in addition to one copy of this fundamental unit a certain amount of DNA indicated by the $^+$ symbol, representing nucleic acid already synthesized in the duplication of this unit. Completion of this duplication is

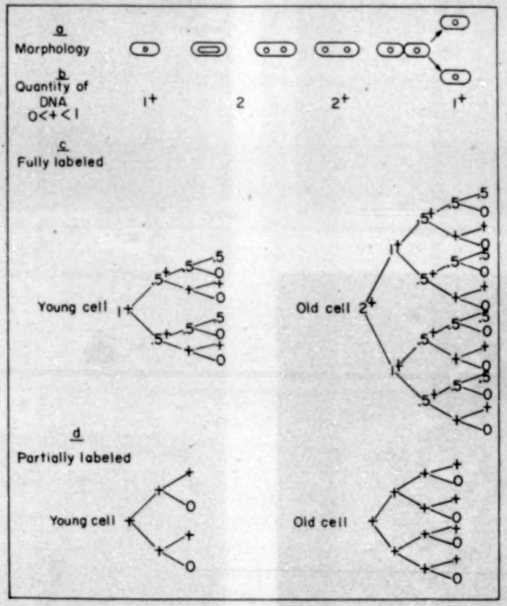

Fig. 3. Schematic representation of: a, the growth; b, DNA content and possible distributions of labeled DNA among progeny of c, fully-labeled cells and d, cells labeled for only part of a generation time.

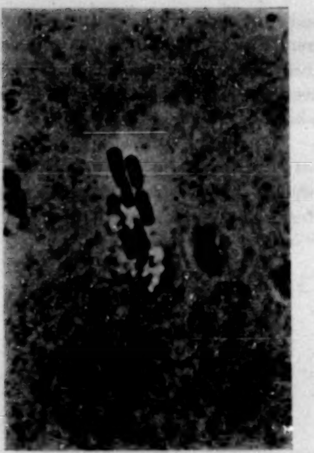

Fig. 4a.

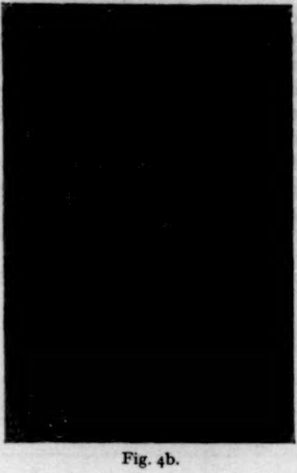

Fig. 4b.

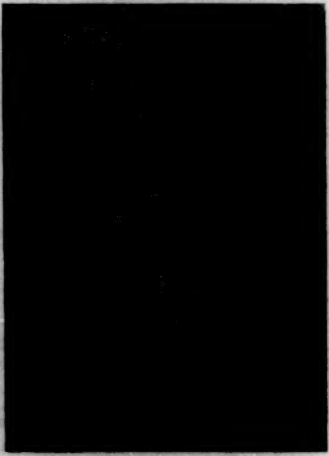

Fig. 5a.

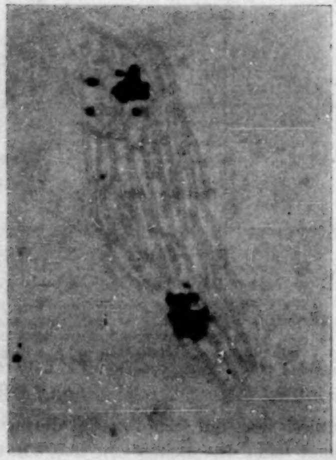

Fig. 5b.

symbolized by the number 2. Because of the continuous nature of DNA synthesis, each of these two units is visualized as proceeding immediately with its replication and concomitant DNA synthesis, symbolized by 2^+, prior to cell division.

In terms of this model, we have depicted the hypothetical results to be expected in a study of the progeny distribution of labeled DNA. The case of fully-labeled cells is contrasted with that of cells labeled for a short time, Fig. 3c and d. For these

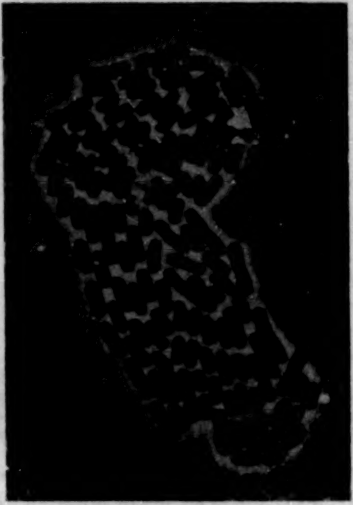

Fig. 6a.

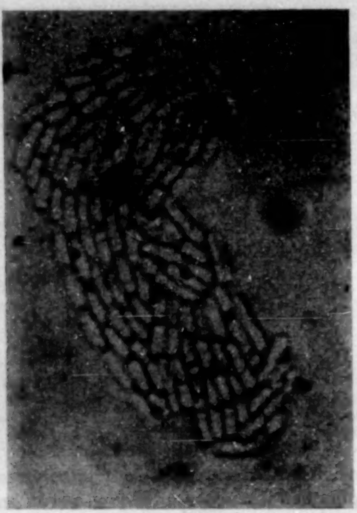

Fig. 6b.

Fig. 7a.

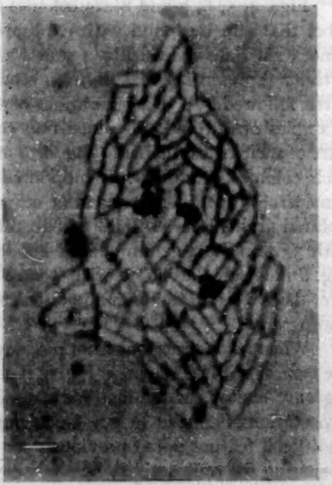

Fig. 7b.

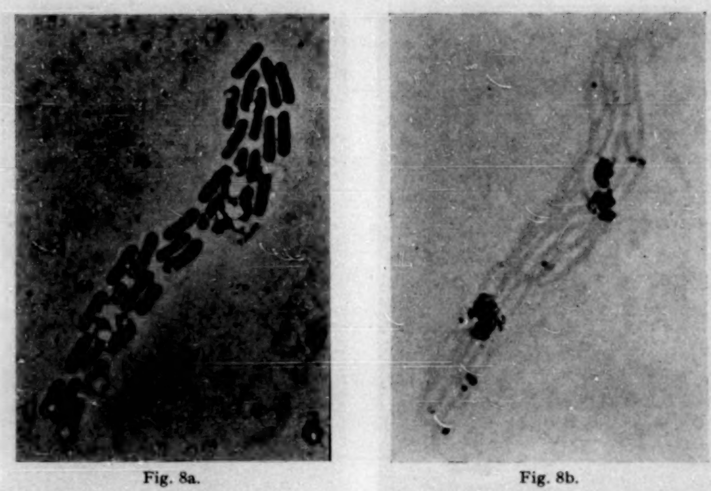

Fig. 8a. Fig. 8b.

Figs. 4–8. Autoradiographs of colonies of *E. coli* 15T$^-$ grown from single cells labeled with [^{3}H]-thymidine for one-half of a generation-time or less. See text for description. a, phase; b, brightfield.

predictions, we have further assumed that the fundamental unit is bipartite and the two subunits are required to separate in replication. Incomplete subunits containing label are represented simply as $^+$ without regard to relative amounts of label.

Figs. 4, 5, 6, 7 and 8 are colonies representative of the types of distribution of label that can be found with exposure to radioactive thymidine for one-half of a generation time or less. Fig. 4 shows a 4 to 8-cell colony with two major clusters of activity representing first division separation of two labeled DNA-containing structures, followed by 1 to 2 generations of all-nothing segregation. Figs. 5 and 6 illustrate examples of 6th and 7th generation colonies respectively each revealing the major part of their tritium label in the form of two clusters of grains. Besides the hot-spots, such colonies contain some individual grains, the numbers of which have varied considerably in different parts of our experiments. We shall say more about these isolated grains later. In addition to colonies with two hot-spots, there occur others with 3, 4 and larger numbers of hot-spots. Fig. 7 is an example of one with three. Fig. 8 illustrates a colony with 4 such spots; these are grouped, however, in pairs over daughters at the fourth or fifth generation (one pair is not well resolved in the photograph).

When reconstructions from progeny-line experiments are made, the distributions are typical of those shown in Fig. 9, if one eliminates from consideration cells containing only one or two grains. With respect to hot-spots, the distributions follow the expectations depicted in our model for cells early in their division cycle with one additional feature noted previously for fully-labeled cells. There is some probability of sharing the radioactivity between daughters at divisions after the first. The model we have suggested would predict an overall simplification of the distributions by

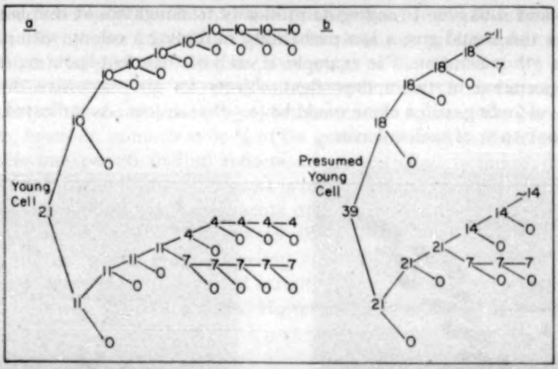

Fig. 9. Progeny-line distributions of hot-spots of incorporated [^{3}H]thymidine from single cells of *E. coli* 15T$^-$ labeled for less than 0.5 generations. a, reconstructed from micromanipulation data; b. probable reconstruction from colony of Fig. 8. Often the location of cells with respect to one another in small colonies permits reconstruction of sibling relationships for several generations.

limiting the time on label. This picture is sustained by the data given in Table I. This shows rather crude frequency distributions of the number of obvious individual hot-spots in randomly selected colonies derived from cells labeled for 5.5 and 0.5 generations respectively. These were approximately 7th or 8th generation colonies in each case. Since there was no selection of the initial cells giving rise to these colonies, the spread in numbers of hot-spots per colony reflect in part the spread in initial complexity attendant on the age distribution of the starting cells. Attention is called to the fact that in spite of this complexity approximately 10 % of the colonies labeled for 0.5 generations contain only two hot-spots.

The conclusions we feel can be drawn from these observations are the following. (a) In these thymine-requiring strains of *E. coli* there exist large DNA-containing structures which can remain intact for many successive replications. (b) The youngest cells of *E. coli* 15T$^-$ contain two of these structures. (c) These two structures have the further property that they must separate from each other at division. (d) These structures are subject to an undetermined probability of fragmentation during the cell division cycle.

Regarding the last conclusion, we specifically consider the sharing of label by daughter cells (at divisions after the 1st for young cells labeled for less than one division, or after the 2nd for young fully-labeled cells) to result from some form of disruption in the continuity of the DNA-containing structure. The alternative that there exists in the initial hot cell more than two objects which are physically in-

TABLE I

DISTRIBUTION OF COLONIES WITH A GIVEN NUMBER OF HOT-SPOTS

Labeled for	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Total
5.5 generations	0	0	0	2	12	17	19	16	11	4	6	4	2	2	3	98
0.5 generations	1	0	11	26	25	15	13	6	8	0	0	3	0	0	0	108

dependent and thus able to segregate randomly to daughters at division seems unlikely, since this would give a low probability of finding a colony with just two hot cells at the 7th generation. For example, if each of these hot-spots represented the random association of two independent objects for six successive divisions, the probability of finding such a clone would be $(0.5)^{12} = 1/4096$. As indicated previously, we find about 10 % of such colonies.

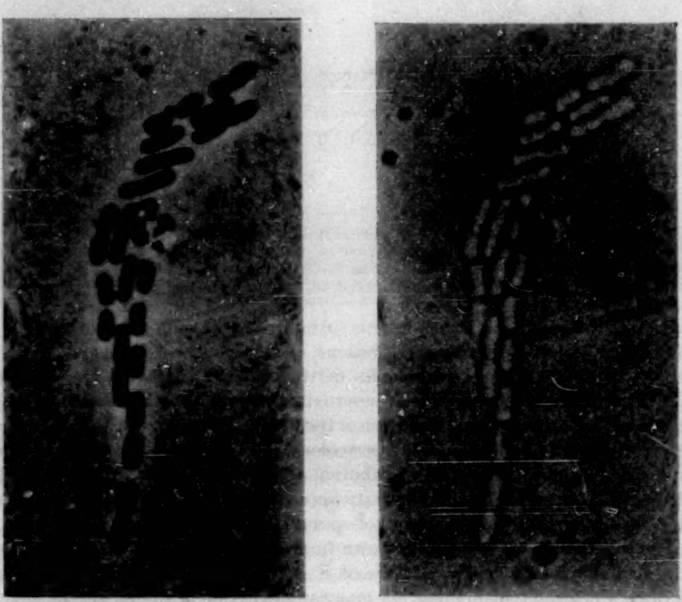

Fig. 10. Autoradiograph of a colony of *E. coli* 15T⁻ grown from a single cell partially labeled with [^{3}H]thymidine showing only one cluster of grains. a, phase; b, brightfield.

A search was made among colonies from partially-labeled cells for any containing only one hot-spot. A few rare examples could be found (Fig. 10). A possible explanation of these in terms of our previous discussion of the relation of DNA synthesis to the division cycle can be ventured.

Occasionally cells may occur whose morphological division is more closely correlated with the completion of synthesis of DNA-containing substructures than is true for most cells. If the time of removal from label was coincident with cell division in such cases and the daughters of such a division became separated during the washing procedures, they could give rise to one-hot-spot clones. This possibility, plus the rarity of such clones, renders more extensive speculation on their significance inadvisable without further data. The occasional occurrence of second division all-nothing distribution in clones from fully-labeled cells are subject to analogous interpretation.

As noted previously, the colonies and progeny-lines from partially labeled cells show a variable number of isolated grains in their autoradiographs. In our first experiments with fully-labeled cells, the percentage of the total activity represented by this non-clustered activity was small enough (around 25 %) that it was construed simply as part of the general pattern of distribution. In some of the partial labeling experiments, however, as much as 80 % of the grains appeared in non-clustered form. Whether this unexpected finding reflects an uncontrolled technical difficulty or represents something of biological interest is not yet clear. Since hot-spots have been a common feature of all our experiments and since we have examples of partial labeling experiments in which almost all the activity is clustered, we have felt justified in discussing the partial labeling experiments with consideration of the hot-spots only. Some of the distributed activity undoubtedly arises from asymmetric fragmentation of the DNA-structures. However, the variability in amount of this activity confuses attempts to estimate the fragmentation frequency per division.

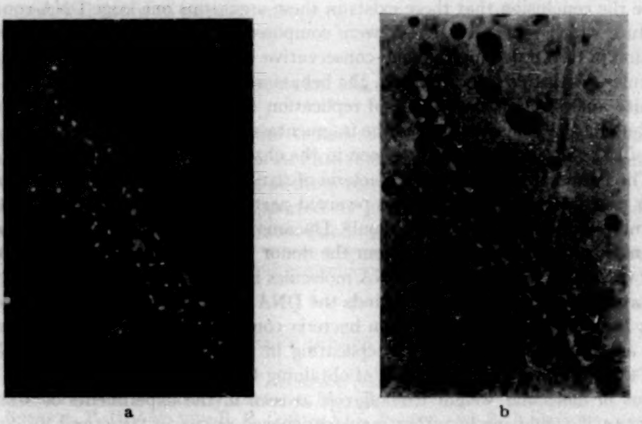

Fig. 11. Autoradiograph of a colony of a uracil-requiring *E. coli* strain grown from a single cell fully-labeled with [^{3}H]uridine. a, phase; b, brightfield.

In the schematic diagram of Fig. 3, depicting the morphology on M-9 medium, we have shown a young cell as possessing one nuclear body. It should be pointed out that on agar containing nutrient broth, cells of *E. coli* 15T$^-$ grow longer than on M-9 and young cells usually contain at least two nuclear bodies. This accounts for the longer cells seen in the photographs.

No easily detectable changes in the essential features of the hot-spot distribution of label have been found to occur over the 9-fold range of specific activity of thymidine used. The results also have seemed relatively invariant to changing the nutrient of the agar medium to M-9, the presence of different quantities of cold thymidine in the agar medium and the temperature of growth on agar. Fig. 11 shows a colony derived from a cell fully-labeled with [^{3}H]uridine. The more random distribution without readily detectable hot-spots is in strong contrast to the thymidine experiments.

DISCUSSION

The young small cell of *E. coli* $15T^-$ grown on simple mineral medium seems to synthesize DNA as if this substance were organized into two large structures. Whether these two units are initially part of one larger unit which replicates in a semi-conservative[8] fashion or whether each represents the conservative replication of two separate structures cannot be decided from the experiments. The light-microscope appearance of one nuclear-body and the electron microscope picture[9] of one continuous structure can at best be considered weak evidence for the first interpretation. MESELSON AND STAHL[10] have reported experiments which indicate that the individual molecules of *E. coli* B replicate as if they consisted of two subunits. The hot-spots in our experiments indicate the presence of structures which are the order of 100 times larger than the molecules observed in the centrifuge experiments. If the results of the density-gradient experiments should prove true also of *E. coli* $15T^-$, it would be difficult to escape the conclusion that there exists in these organisms one large DNA-containing structure which replicates as if it were composed of two subunits, this form of replication in turn reflecting the semi-conservative form of replication of the individual molecules. With this interpretation, the behavior of these bacterial structures would be quite analogous in their mode of replication to plant chromosomes as shown by TAYLOR *et al.*[11, 12]. One wonders if the fragmentation we see might also be the counterpart of sister-strand crossing-over seen in the chromosomal studies.

The notion of the presence in bacteria of structures analogous to chromosomes of higher organisms is not new. It has received particular support in recent times from the studies of bacterial recombination[13]. The amount of the material and the manner of transfer of genetic characters from the donor to the recipient cell would seem to imply the organization of many DNA molecules into a larger linear superstructure[14]. The nature of the material which binds the DNA together is not known. The experiments both on chromosomes and on bacteria could be explained by the presence of DNA as one large supermolecule replicating in the manner suggested by WATSON AND CRICK[15]. The comparative ease of obtaining DNA preparations relatively homogeneous in molecular weight from *E. coli* as seen in the experiments of MESELSON AND STAHL[10] would imply rather regularly spaced points of structural weakness in such a super-molecule. It seems more attractive to think that some other material serves to bind similarly-sized DNA molecules together. Whatever the nature of this linkage, it must allow the semi-conservative replication of individual molecules to be reflected in the mode of replication of the superstructure. Some models suggesting how this might occur have been discussed previously[11, 16]. Reports in the literature[17–19] of the isolation of DNA-containing structures from bacteria lend hope for the characterization of the superstructure(s).

The fragmentation we have observed could conceivably occur within DNA molecules, in the material that may link them, or at the junctions between the two. If the first were true, and breakage of one molecule were equivalent to the observed event, the frequency of fragmentation is low enough and the size of the large structures is large enough that at most 1–2 % of the cell's DNA molecules would be broken in one division cycle. It is doubtful if these would have been detected in the gradient-density experiments of MESELSON AND STAHL.

Studies on the organization and mode of replication of the DNA of virulent bacteriophage have presented somewhat conflicting results[20]. More recent experiments indicate that some of the DNA molecules of these viruses may be linked in a manner formally similar to bacteria.

The possibility that the radiation associated with tritium-decay during the experiment influences the results cannot be evaluated unequivocally at present. Artificial aggregation of DNA by the radiation would seem unlikely in view of the range of specific activities employed, unless the dose-response curve for such a phenomenon were quite peculiar. Progeny-line experiments show that any such hypothetical aggregation is at least compatible with normal growth patterns. The fragmentation might result from the radiation, but again the lack of obvious gross changes in the distribution patterns at different specific activities does not favor this idea.

The generality of these results for non-mutants and other species of bacteria needs exploration. It is not inconceivable that under different circumstances, the bacterial chromosomes will prove to be more ephemeral.

ACKNOWLEDGEMENTS

We express our appreciation for the technical assistance of Mrs. M. MUELLER. Especially, we are indebted to Dr. R. B. PAINTER for his personal generosity and his helpful discussions during the course of the work.

This investigation has been supported by a research grant (C 3860) from the National Cancer Institute, United States Public Health Service.

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METABOLISM OF URACIL AND 5-FLUOROURACIL BY DRUG-SENSITIVE AND BY DRUG-RESISTANT BACTERIA

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(Received September 7th, 1959)

SUMMARY

Escherichia coli resistant to growth inhibition by 5-fluorouracil has lost the capacity to form 5′-ribonucleotide derivatives of uracil and 5-fluorouracil. Enzyme preparations from the resistant *E. coli* cells failed to catalyze the reactions of uracil and 5-fluorouracil with 5-phosphoribosyl-1-pyrophosphate, whereas similar enzyme preparations from the parent drug-sensitive *E. coli* did catalyze these reactions.

These findings suggest that one mechanism of resistance to 5-fluorouracil, at least in the case of the highly resistant bacterial cells studied, is of the same type previously shown to exist for 8-azaguanine and 6-mercaptopurine—namely, failure to form fraudulent ribonucleotide derivatives from the corresponding base.

INTRODUCTION

Presently available evidence shows that a major biosynthetic pathway by which purine bases are converted to ribonucleotides is by reaction with 5-phosphoribosyl-1-pyrophosphate (PRPP)[1,2]. WAY AND PARKS[3,4] have shown that purine analogs, such as 8-azaguanine, 6-mercaptopurine and pyrazolo(3,4-*d*)pyrimidines, react with PRPP in the presence of enzymes from mammalian liver to yield fraudulent purine ribonucleotides. LUKENS AND HERRINGTON[5] have also studied the enzyme-catalyzed reaction of 6-mercaptopurine with PRPP.

In a comparison of the metabolism of purine analogs by actively growing bacterial and neoplastic cells that were sensitive or resistant to 8-azaguanine and 6-mercaptopurine, it was observed that the drug-resistant cells failed to form significant amounts of ribonucleotide derivatives of these analogs as compared to the drug-sensitive cells[6,7]. It has also been demonstrated that enzyme preparations from drug-resistant bacterial and neoplastic cells lack the capacity to catalyze the reaction of certain of the purines and of the relevant purine analogs with PRPP to yield ribonucleotides[8,9]. On the basis of these findings, it was postulated that fraudulent purine ribonucleotides, such as 8-azaguanylic acid and 6-mercaptopurine ribonucleotide or

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Abbreviations: U, uracil; UMP, uridine-5′-monophosphate; UDP, uridine-5′-diphosphate; UTP, uridine-5′-triphosphate; UDPG, uridine-5′-diphosphate glucose; UDPAG, uridine-5′-diphosphate-N-acetylglucosamine; 5-fluorouracil analogs are abbreviated as FU, *etc.*; CMP, cytidine-5′-monophosphate; CDP, cytidine-5′-diphosphate; CTP, cytidine-5′-triphosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; Tris, tris(hydroxymethyl)aminomethane–HCl buffer.

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derivatives of these compounds, were active inhibitors and that one mechanism of resistance to these analogs was the deletion or inactivation of enzymes catalyzing the lethal synthesis of these fraudulent ribonucleotides.

It was considered of interest to extend this work to include studies of resistance to pyrimidine analogs. It has been demonstrated that pyrimidine nucleotides are formed from orotidylic acid following the condensation of orotic acid with PRPP[10]. Recently CRAWFORD *et al.*[11] and CANELLAKIS[12] have shown that uridine-5'-phosphate can be formed by direct condensation of uracil with PRPP and that the enzyme catalyzing this reaction is distinct from orotidylic acid pyrophosphorylase[11]. This route of uridylic acid synthesis is distinct from the uracil to uridine[13, 14] and uridine to uridylic acid[12] pathway demonstrated in bacterial and mammalian cells and recently investigated by REICHARD AND SKÖLD[15] in ascites tumor cells.

In the light of these findings, we were led to study the metabolism of uracil and 5-fluorouracil in bacteria that are sensitive or resistant to 5-fluorouracil, a compound which possesses interesting anticancer and antibacterial activities[16, 17].

METHODS

Materials

The 5-fluoropyrimidine derivatives used in this study were obtained from Hoffmann-LaRoche Inc., Nutley, N. J., through the generosity of Dr. ROBERT DUSCHINSKY. [2-^{14}C]Uracil (2 mC/mmole) and 5-[2-^{14}C]fluorouracil (2.5 mC/mmole) were purchased from Isotopes Specialties Company, Burbank, Calif. Pyrimidine nucleosides and nucleotides, UDPG, and the magnesium salt of PRPP were purchased from Pabst Laboratories, Milwaukee, Wisc. A sample of uridine diphosphate N-acetylglucosamine was obtained from Dr. ELIZABETH S. MAXWELL, National Institutes of Health, Bethesda, Md.

Microorganisms

Escherichia coli (American Type Culture Collection 9637) was the parent drug-sensitive organism from which a 5-fluorouracil-resistant strain, designated *E. coli*/FU, was isolated by serial transfer in minimal medium[18] containing increasing concentrations of 5-fluorouracil. The resistant organism used in this study reached maximum growth comparable to that of the parent strain on overnight incubation at 37° in medium containing 5-fluorouracil (1000 μg/ml; 7.6 μmoles/ml). It was observed that growth of the parent *E. coli* 9637 was completely inhibited by 1.0 μg/ml (0.007 μmoles/ml) of 5-fluorouracil.

In the growth inhibition studies with 5-fluoropyrimidines (Table I), solutions of the compounds were sterilized by filtration and added aseptically to tubes of minimal medium[18]. Growth was measured by optical density determinations at 660 mμ in the Bausch and Lomb Spectronic 20 photoelectric colorimeter.

Pyrimidine metabolism

The intermediary metabolism of [2-^{14}C]uracil and 5-[2-^{14}C]fluorouracil was studied in exponentially growing cultures of *E. coli* 9637 and *E. coli*/FU. A standard inoculum was prepared from 18-h cultures of saline-washed cells. The initial optical density of the medium was adjusted to approximately 0.2. The inoculated medium

TABLE I

EFFECT OF 5-FLUOROPYRIMIDINES AND DERIVATIVES ON THE GROWTH OF DRUG-SENSITIVE AND DRUG-RESISTANT *Escherichia coli**

Inhibitor	*Concentration for 50 % inhibition (μmoles/ml)*		*Cross resistance index***
	E. coli *9637*	E. coli/*FU*	
5-Fluorouracil	0.005	> 5.0	> 1000.0
5-Fluorouridine	0.005	> 1.0	> 200.0
5-Fluoro-2′-deoxyuridine	0.006	0.1	16.5
5-Fluorocytosine	2.1	> 5.0	> 2.4
5-Fluorocytidine	0.004	> 1.0	> 250.0
5-Fluoro-2′-deoxycytidine	0.007	1.0	143.0
5-Fluoroörotic acid	0.07	0.8	11.4

* Growth after 16 h incubation at 37° was measured in the Bausch and Lomb Spectronic 20 as optical density at 660 mμ.

** Cross resistance index = $\frac{\text{concentration of inhibitor for 50\% inhibition of } E.\ coli/\text{FU.}}{\text{concentration of inhibitor for 50\% inhibition of } E.\ coli \text{ 9637.}}$

was then divided into 65-ml portions in 300 ml Erlenmeyer flasks fitted with 18 × 160 mm culture tubes by means of a ground-glass joint. The flasks, which were mechanically agitated in a water bath at 37°, could be inverted and the optical density of the medium conveniently determined at intervals in order that growth curves could be plotted. The incubation flasks were fitted with sidearms to permit convenient addition of radioactive substrates and to admit air through sterile cotton plugs. After 120 min incubation of the cultures, the optical density approached 0.7 and radioactive substrates were added; maximum growth of control cultures (optical density of approximately 1.0) was reached about 180 min after inoculation under these conditions. The generation times of *E. coli* 9637 and of *E. coli*/FU were 50 min and 54 min, respectively, under these experimental conditions. 15 Min after the radioactive substrates were added, growth was stopped by pouring the cultures on 35 g of ice. Cells were removed by centrifugation and were then re-suspended in 10 ml of ice water. The cell suspension was immediately poured into 40 ml of boiling ethanol and extracted for 5 min. Cell debris was removed by centrifugation; the clear supernatant solution thus obtained was concentrated to a small volume by evaporation under reduced pressure and was then lyophilized. The residue was dissolved in approximately 0.5 ml of water; the exact volume of water used was proportional to the optical density of the culture when it was poured on ice. In this manner comparable cell extracts were prepared for paper chromatography. The chromatographic-radioautographic analysis of such extracts has been described[7, 19].

A study of the incorporation of [2-^{14}C]uracil and of 5-[2-^{14}C]fluorouracil into the nucleic acids of *E. coli* 9637 and of *E. coli*/FU was made. To 500 ml cultures of bacteria incubated at 37° in a shaking incubator was added 8.75 μC of radioactive substrate. When maximum growth was reached approximately 4 h later the cells were separated by centrifugation and extracted with hot aqueous ethanol as described above. The cell debris served as the starting point for the isolation of the nucleic acid by procedures previously described[7]. Specific activities (μC/100 μg) of the combined nucleic acid (RNA + DNA) thus obtained were determined by evaporating solutions of nucleic acid on planchets and counting in gas flow proportional counters (Table II).

TABLE II

INCORPORATION OF [2-^{14}C]URACIL AND 5-[2-^{14}C]FLUOROURACIL INTO THE NUCLEIC ACID OF *Escherichia coli*

Combined nucleic acids from:*	*Specific activity of nucleic acid** (counts/sec/100 μg)*	
	[2-^{14}C]Uracil	*5-[2-^{14}C]Fluorouracil*
E. coli 9637	348	287
E. coli/FU	< 1	< 1

* Combined nucleic acids (CNA) = RNA + DNA.

** Cultures of *E. coli* were incubated in the presence of the radioactive substrates for approximately 4 h. Alkaline hydrolysis of CNA followed by electrophoretic analysis of ribonucleotides demonstrated the presence in RNA of uridylic and cytidylic acids derived from [2-^{14}C]uracil and of 5-fluorouridylic acid derived from 5-[2-^{14}C]fluorouracil.

Alkaline hydrolysates of nucleic acid were analyzed by paper electrophoresis in ammonium formate buffer (pH 3.5); radioactive pyrimidine ribonucleotides were detected by exposing the electrophoresis strips to X-ray film.

Enzyme preparations

Soluble enzyme preparations were made from cells harvested by centrifugation from 500-ml cultures of *E. coli* 9637 and *E. coli*/FU grown for 18 h at 37°; aeration of the cultures was provided by mechanical agitation. The cells were washed twice in physiological saline, suspended in 10 ml of 0.2 *M* Tris buffer (pH 7.6) and then subjected to sonic vibration* at 0° for 5 min. The supernatant solution obtained after centrifugation of the resulting suspension at 25,000 × *g* for 1 h at 4° served as the soluble enzyme preparation. Protein content of such enzyme preparations was determined by the OYAMA-EAGLE modification[20] of the method of LOWRY *et al.*[21] using crystalline bovine albumin (Armour and Co.) as a reference standard**. Similar enzyme preparations in 0.1 *M* phosphate buffer (pH 7.2) were also made; these were comparable in activity to the Tris buffer preparations. After reaction of pyrimidines or purines with PRPP in the presence of the enzyme preparations the incubation medium was analyzed by paper chromatography to separate the pyrimidine or purine bases from the nucleotides. The % conversion of base to nucleotide was determined by eluting the radioactive compounds, evaporating the eluates on steel planchets, and quantitatively measuring the activity in internal gas-flow proportional counters.

RESULTS

Response of E. coli *9637 and* E. coli*/FU to 5-fluoropyrimidine derivatives*

It was observed that 5-fluorouracil, 5-fluorouridine, 5-fluoro-2′-deoxyuridine, 5-fluorocytidine, and 5-fluoro-2′-deoxycytidine were completely inhibitory to the growth of *E. coli* 9637 at low concentrations (Table I). Higher levels of 5-fluoroörotic acid were required for complete inhibition; 5-fluorocytosine was a poor growth inhibitor. Growth of the 5-fluorouracil-resistant organism (*E. coli*/FU) was inhibited by the other 5-fluoropyrimidine compounds at concentrations higher than those required to inhibit *E. coli* 9637 (Table I). It is of interest that 5-fluoro-2′-deoxyuridine proved to be the most effective inhibitor of *E. coli*/FU in these experiments.

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* Raytheon 9-kc, 50-W Magnetostriction Oscillator, Raytheon Corp., Waltham, Mass.

** A Tris buffer blank was used in the protein determinations to compensate for the color developed by Tris with the reagents used.

Metabolism of [2-^{14}C]*uracil and* 5-[2-^{14}C]*fluorouracil by sensitive and 5-fluorouracil-resistant microörganisms*

It was found that [2-^{14}C]uracil was extensively metabolized to nucleotide derivatives in 15 min by exponentially growing cultures of the parent drug-sensitive *E. coli* 9637 but not by *E. coli*/FU (Fig. 1). Similarly, 5-[2-^{14}C]fluorouracil was metabolized to nucleotide derivatives by sensitive but not by resistant cells (Fig. 1). It was also observed that *E. coli* 9637 incorporated significant amounts of [2-^{14}C]-uracil and of 5-[2-^{14}C]fluorouracil into its nucleic acid (Table II). Incorporation into RNA of [2-^{14}C]uracil as uridylic and cytidylic acids and of 5-[2-^{14}C]fluorouracil as 5-fluorouridylic acid was shown by electrophoretic analysis of alkaline hydrolysates of nucleic acid samples. No 5-fluorocytidylic acid could be detected. There was no significant incorporation of uracil or of 5-fluorouracil into the nucleic acids of *E. coli*/FU (Table II).

A comparison of the metabolism of [2-^{14}C]uracil and 5-[2-^{14}C]fluorouracil by exponentially growing *Streptococcus faecalis* that are sensitive (SF/O), or resistant (SF/FU), to growth inhibition by 5-fluorouracil gave similar results. Thus, [2-^{14}C]-uracil and 5-[2-^{14}C]fluorouracil were extensively metabolized to nucleotides by SF/O but were not converted to nucleotide derivatives by SF/FU*. These results are analogous to those obtained in the study of resistance to 8-azaguanine and 6-mercaptopurine[7].

The pyrimidine nucleotides were not adequately resolved by the two-dimensional chromatographic system used (phenol–water : *n*-butanol–propionic acid–water). However, one-dimensional chromatography of *E. coli* 9637 extracts in isobutyric acid–ammonium isobutyrate[22] or in ethanol–ammonium acetate[23,24] showed a number of separate radioactive spots derived from either [2-^{14}C]uracil or 5-[2-^{14}C]-fluorouracil. These derivatives behaved chromatographically like uracil, UMP, UDP, UTP, UDPG and UDPX, where X is probably N-acetylglucosamine (Fig. 2). In all cases the 5-fluorouracil derivatives behaved chromatographically like those of uracil. Further evidence for the identity of the [2-^{14}C]uracil and 5-[2-^{14}C]fluorouracil derivatives was obtained by chemical and enzymic hydrolysis of the nucleotides. Hydrolysis by 1 *N* hydrochloric acid at 90° for 30 min converted the di- and tri-phosphate derivatives (including the nucleoside diphosphate glycosyl derivatives) to compounds chromatographically identical with uridylic acid and 5-fluorouridylic acid. Intestinal phosphatase hydrolyzed urididylic and 5-fluorouridylic acids to the corresponding nucleosides. The radioactive nucleoside derived from UMP was identified as [2-^{14}C]uridine by co-chromatography with uridine in several solvent systems and by co-electrophoresis with uridine in borate buffer. Similarly, 5-fluorouridine was identified as the radioactive nucleoside derived from FUMP.

The compounds derived from [2-^{14}C]uracil and 5-[2-^{14}C]fluorouracil which

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* **Unpublished observations, C. Sparks, P. Stutts and R. W. Brockman, Southern Research Institute.**

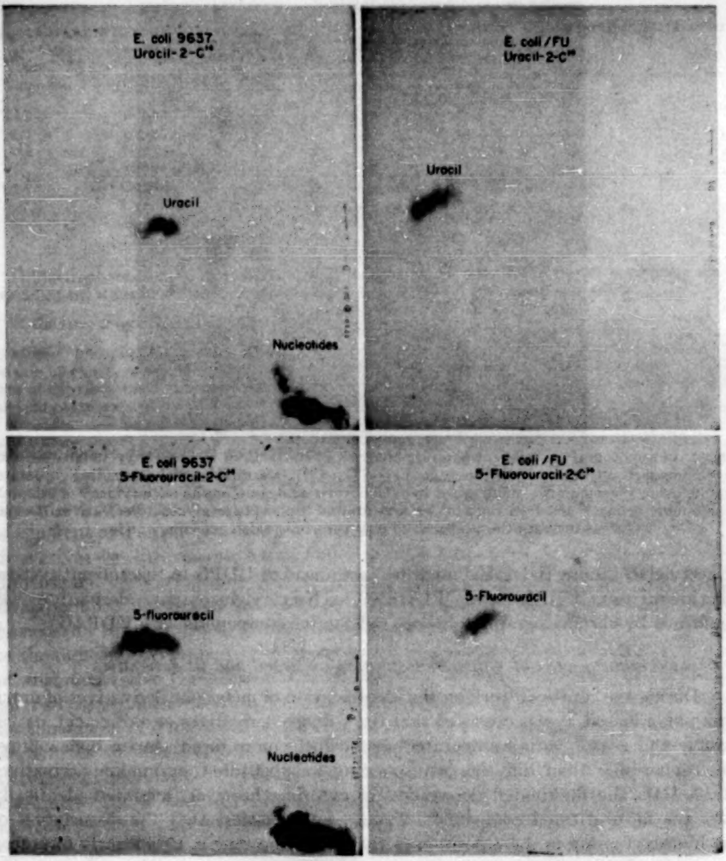


Fig. 1. Metabolism of [2-^{14}C]uracil and 5-[2-^{14}C]fluorouracil by 5-fluorouracil-sensitive (9637) and -resistant (FU) *Escherichia coli*. Extracts of cells were analyzed by paper chromatography in two dimensions: phenol–water (←) followed by *n*-butanol–propionic acid–water (↑). Chromatograms were exposed for 2 weeks to X-ray film (Eastman Kodak single-emulsion, blue-sensitive, 14 × 17″) to detect radioactive compounds.

migrated like UDPG were eluted from the one-dimensional chromatogram developed in ethanol–ammonium acetate (Fig. 2). Both of these radioactive derivatives migrated with a known sample of UDPG upon co-chromatography in ethanol–ammonium acetate.

The radioactive "UDPX" and "FUDPX" derivatives migrating just ahead of UDPG upon chromatography in ethanol–ammonium acetate (Fig. 2) are probably N-acetylglucosamine derivatives. LELOIR *et al.*[23,24] reported that uridine diphosphate

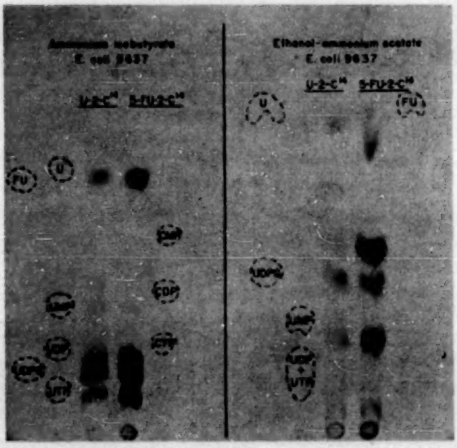


Fig. 2. Chromatographic-radioautographic analysis of extracts of *E. coli* 9637 incubated with [2-^{14}C]uracil (U-2-C^{14}) or 5-[2-^{14}C]fluorouracil (5-FU-2-C^{14}). One-dimensional descending chromatography using Whatman No. 3 MM paper with isobutyric acid–ammonium isobutyrate[22] or ethanol–ammonium acetate[23] resolved nucleotides and uridine diphosphate glycosyl derivatives. Dotted areas indicate the positions of u.v.-absorbing reference compounds.

N-acetylglucosamine (UDPAG) migrates just ahead of UDPG in this solvent system. The identity of "UDPX" and "FUDPX" as N-acetylglucosamine derivatives was confirmed by chromatography of these radioactive compounds with UDPAG.

A physicochemical effect of 5-fluoro-substitution on uracil and its derivatives

During the course of work on the identification of metabolic derivatives of uracil and 5-fluorouracil, it was observed that the 5-fluoro derivatives of orotic acid, uracil, uridine and 2′-deoxyuridine migrated significantly more rapidly upon high voltage electrophoresis[25] than did the corresponding unsubstituted pyrimidine derivatives (Table III). The fluorinated derivatives of cytosine, however, migrated identically with the unsubstituted compounds. These results suggest that the dissociation of the hydroxyl group in the 4-position of the pyrimidine ring is significantly increased as a result of the influence of the fluorine substituent in the 5-position. CHAUDHURI, MONTAG AND HEIDELBERGER[26] observed that 5-fluoro-substituted pyrimidines were more acidic than the corresponding pyrimidines upon ion-exchange chromatography. It is also of interest that GINER-SOROLLA AND BENDICH[27] observed a marked increase in the acid strength of 6-methyluracil upon replacement of the hydrogen atoms of the methyl group by fluorine atoms. DAHL, WAY AND PARKS[28] recently observed that orotic and 5-fluoroörotic acids have similar Michaelis constants when compared as substrates for yeast orotidylic acid pyrophosphorylase. These authors also observed that the pH optima for nucleotide formation are similar and that 5-fluoroörotic acid reacts about twice as rapidly as does orotic acid. The substitution of the fluorine atom on the pyrimidine ring alters both the physico-chemical properties of the molecule and the rate of an enzyme catalyzed reaction.

TABLE III

ELECTROPHORETIC MIGRATION OF PYRIMIDINE DERIVATIVES: EFFECT OF 5-FLUORO-SUBSTITUTION[*]

	Relative migration distance of pyrimidine derivatives[**]	
	Unsubstituted compound	*5-Fluoro-substituted compound*
Uracil	30	82
Uridine	66	85
2′-Deoxyuridine	18	60
Cytosine	— 5	— 4
Cytidine	46	42
2′-Deoxycytidine	—12	—12
Orotic acid	97	113

[*] Electrophoresis on Whatman No. 3 MM paper was carried out in apparatus similar to that described by MARKHAM[25]. Sodium tetraborate (0.05 M; pH 9) served as the buffer.

[**] Relative migration distance $= \dfrac{\text{migration distance (cm) of pyrimidine}}{\text{migration distance (cm) of uridine-5'-phosphate}} \times 100$

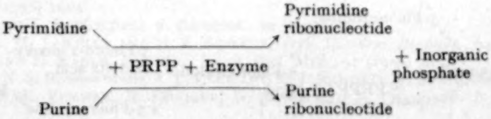
Uridine-5′-phosphate generally moved 14 to 16 cm from the origin upon electrophoresis for 90 min at 750 V. A negative sign indicates migration toward the cathode. The difference in migration of the ribo- and deoxyribonucleosides is apparently due to the formation by the former of an ionized complex with borate through the 2′,3′-*cis*-glycol structure[29].

Electrophoretic analysis of pyrimidine nucleoside derivatives

The electrophoretic mobilities of the pyrimidine compounds and the 5-fluoroderivatives are given in Table II. The separation of ribo- and deoxyribonucleosides[29] is apparent and an effort was made to detect the presence of 5-fluoro-2′-deoxyuridine derivatives by this means. All of the nucleotide derivatives eluted from the two-dimensional chromatograms (Fig. 1) were completely hydrolyzed to nucleoside derivatives upon incubation overnight at 37° with snake venom (*Crotalus atrox*)[*]. The nucleosides thus formed were subjected to paper electrophoresis in borate buffer. All of the radioactivity from 5-[2-^{14}C]fluorouracil was contained in 5-fluorouridine; most of the radioactive compounds derived from [2-^{14}C]uracil were present as uridine after snake venom hydrolysis. No radioactive deoxyribonucleosides could be detected; no conversion of fluorinated pyrimidine derivatives to the corresponding pyrimidine derivatives was observed. It is possible that the solvents used in two-dimensional chromatography may have destroyed deoxyribonucleoside derivatives or that the concentrations of such derivatives formed during the 15-min incubation of radioactive substrates with exponentially growing *E. coli* 9637 were too low to be detected.

Studies with enzyme preparations from drug-sensitive and -resistant E. coli

The capacity of enzyme preparations from *E. coli* 9637 and *E. coli*/FU to catalyze the following reactions was studied:



The data summarized in Table IV show that uracil, 5-fluorouracil, orotic acid, and 5-fluoroörotic acid react with PRPP in the presence of enzyme preparations from

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[*] Obtained from Ross Allen's Reptile Institute, Silver Springs, Florida.

E. coli 9637 to afford the corresponding ribonucleotides. Enzyme preparations from *E. coli*/FU failed to catalyze the formation of uridylic and 5-fluorouridylic acids, whereas orotidylic and 5-fluoroörotidylic acids were formed in good yields. The conversion of purines to ribonucleotides, included as a reference reaction, took place equally well in the presence of enzyme preparations from *E. coli* 9637 or *E. coli*/FU.

TABLE IV

FORMATION OF RIBONUCLEOTIDES BY REACTION OF PYRIMIDINE AND PURINE BASES WITH 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE IN THE PRESENCE OF ENZYMES FROM *E. coli* 9637 AND *E. coli*/FU

Biochemical reaction	*Base + PRPP* $\xrightarrow{\text{Enzyme}}$ *Ribonucleotide** *% conversion of base to nucleotide*	
	E. coli 9637	E. coli/*FU*
Uracil ⟶ UMP	11.7**	0
5-Fluorouracil ⟶ 5-FUMP	12.6**	0
Orotic acid ⟶ OMP	53.3**	62.1
5-Fluoroörotic acid ⟶ 5-FOMP	68.7**	92.6
Adenine ⟶ AMP	33.1	36.3
Guanine ⟶ GMP	40.4	47.6
Hypoxanthine ⟶ IMP	55.3	78.7
Xanthine ⟶ XMP	40.1	47.1

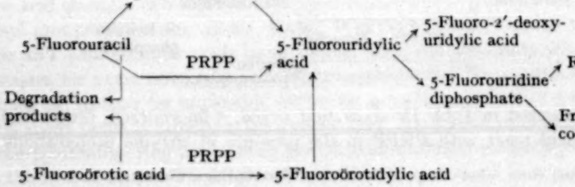
* Experimental conditions: Base (0.5 μmole; 0.2 μC); PRPP (1.0 μmole); enzyme equivalent to approximately 0.3 mg protein; 0.1 *M* Tris buffer (pH 7.6) to a volume of 1 ml. The mixture was incubated for 1 h at 37°.

** These figures are average values from four or more independent experiments. Other data are based on results of assay of the catalytic activity of at least two different enzyme preparations.

DISCUSSION

While the present study was in progress, REICHARD AND SKÖLD[30] described the results of a comparison of the metabolism of uracil in sensitive and 5-fluorouracil-resistant Ehrlich ascites tumor cells. It was demonstrated that the 5-fluorouracil-resistant Ehrlich ascites cells were unable to form 5′-ribonucleotide derivatives of uracil as a result of loss of uridine phosphorylase activity. Uridine kinase activity in the resistant cell line, although lowered, was significant. These results are consistent with the findings of HANDSCHUMACHER[31] recently reviewed by WELCH[32].

From these results, it appears that resistance to 5-fluorouracil can arise by deletion (or inactivation) of uridylic acid pyrophosphorylase, as in the case of *E. coli* in the present study, or by loss of uridine phosphorylase activity as in the case of Ehrlich ascites tumor cells[30]. It appears that 5-fluorouracil is metabolized by the same biosynthetic pathways as is uracil[17, 33]:



In studies with 5-fluorouracil, HEIDELBERGER *et al.*[16, 34] and EIDINOFF *et al.*[35] observed inhibition of the conversion of formate to the methyl group of thymine. COHEN *et al.*[17] have shown that 5-fluoro-2'-deoxyuridylic acid specifically inhibits the synthesis of thymidylic acid in a strain of *E. coli*, thus causing unbalanced growth. It is therefore understandable that failure to form 5-fluorouridylic acid and hence 5-fluoro-2'-deoxyuridylic acid from 5-fluorouracil would be an effective mechanism of resistance. In view of the multiple steps involved in uracil metabolism, multiple sites of inhibition by pyrimidine analogs[17, 33–40] and multiple mechanisms of resistance[41] to such analogs are possible.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. R. E. PARKS, JR., University of Wisconsin, and to Dr. E. P. ANDERSON, National Cancer Institute, for valuable discussions of aspects of this work and to Drs. L. L. BENNETT, JR., and H. E. SKIPPER, Southern Research Institute, for their interest in this study. The work was supported by the Cancer Chemotherapy National Service Center, National Cancer Institute, under National Institutes of Health Contract No. SA-43-pH-1741 and by grants from the Charles F. Kettering Foundation and the Alfred P. Sloan Foundation. We are indebted to Dr. G. J. DIXON and Miss E. A. DULMADGE for the protein assays. Quantitative determinations of radioactivity were made by Miss L. SIMPSON and Miss F. NEWMAN.

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STUDIES ON A PROTEIN-SYNTHESIS-AFFECTING SUBSTANCE FROM BIOLOGICAL MATERIALS

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(Received May 4th, 1959)

SUMMARY

A substance of lipid character, containing carbohydrate and phosphorus, which stimulates the incorporation of labelled amino acids in the protein of Ehrlich ascites cells, was found in animal tissues and egg yolk. The content of the tissues of tumour-bearing individuals was higher than that of the tissues of normal ones. The substance was obtained in crystalline form.

INTRODUCTION

In a recent paper the effect was described of homogenates of various rat tissues on the incorporation of labelled methionine into the proteins of Ehrlich ascites cells *in vitro*[1]. It was found that these preparations exhibit a stimulatory as well as an inhibitory effect on this process, depending on the dose used. It seemed very probable that some substance present in the tissues might be responsible for these effects.

Some indications do already exist that there may be factors in tissues capable of influencing the biosynthesis of proteins[2]. Evidence was obtained[1] that tissues of tumour-bearing animals contain higher amounts of the active substance than those of normal ones. It seems quite reasonable to suppose that these different quantities of a protein-synthesis-affecting material in various tissues may be connected in some way with the varying abilities of these tissues to incorporate labelled amino acids *in vitro*. Such differences are known to exist between tumours and corresponding normal tissues[3] as well as between similar tissues of tumour-bearing and normal animals[4].

It follows from our experiments that factors capable of stimulating the biosynthesis of proteins may possess a more general ability to enhance growth. A wide variety of growth-enhancing factors seems to exist in various tissues[5] and it is quite possible that some of them may exhibit this activity by influencing protein synthesis. This is true for the substance whose isolation will be described in this paper.

In our investigation attempts were made first to isolate the active substance from animal tissues. These attempts were successful but only a very small quantity of this material has been obtained in crystalline form. Egg yolks appeared to be a more suitable material for preparing it[6]. Yolks are known to contain a substance capable of inducing malignant growth[7] that seems to be identical with a growth-promoting factor described by other authors[8]. It seemed very probable to us that this substance may be also identical with that responsible for the stimulation of protein synthesis[1] and enhanced growth of malignant tissues[9]. This possibility was strengthened by the fact that carcinogenic substances seem to affect the protein synthesis in the same dose-dependent way as did tissue homogenates[10].

MATERIALS AND METHODS

Biological materials

Wistar rats of both sexes, weighing 150-200 g, were used. They were fed on a Larsen diet and given water *ad libitum*. Walker 256 carcinoma was implanted subcutaneously. Two weeks after the transplantation of the tumour the animals were killed by a blow on the head and the tissues immediately excised. For preliminary experiments, lyophilized liver tissue from normal rats was used. A 10% homogenate in water was freeze-dried by a method described elswhere[11]. The lyophilized tissue was stored in a deep-freezing unit (—30°) and used after 7 months of storage.

Mice, from which tissues for some preparations originated, were of a mixed albino stock, weighing 20-25 g, of both sexes. They were maintained on Larsen diet and given water *ad libitum*. Animals bearing subcutaneous grafts of Sarcoma 37 were used 2 weeks after implantation.

The Ehrlich ascites carcinoma, used for the study of incorporation of labelled amino acids into its proteins, was a frozen preparation as described in our previous paper[1].

Fresh fertilized eggs from white Leghorn hens were used as starting material for preparation of the active substance from yolks.

Chemicals

All chemicals used in our experiments were of A.R. grade. All solvents were redistilled before use. The petroleum ether used was a fraction boiling between 60 and 80°. Alumina used for column chromatography was standardized according to BROCKMANN[12]. To obtain the highest activity grade, alumina was heated for at least 5 h at 300° in an iron dish and stored immediately under petroleum ether. All preparations of alumina were tested for their activities according to BROCKMANN[12].

Labelled amino acids used in our experiments were ^{35}S-labelled *dl*-methionine, with an average activity of 0.6–0.7 mC/mg, and ^{14}C-carboxyl-labelled glycine, with an average activity of 0.16 mC/mg. Both these compounds were obtained from the Radiological Research Institute in Prague. A solution of 1.5 mg methionine in 100 ml of an isotonic KCl–$KHCO_3$ mixture (1000:8) and 26 mg glycine in 10 ml of the same solution was used.

Chemical methods

Alumina chromatography was performed under atmospheric pressure in glass tubes of varying diameters and lengths provided with a stopcock.

Paper chromatography was performed using the descending technique. The following solvent systems were used: (1) water-saturated n-butanol, Whatman No. 1 paper, (2) water-saturated collidine, Whatman No. 1 paper, (3) chloroform–methanol (1:9), Whatman No. 3 paper, (4) acetic acid–chloroform–liquid paraffin (65:25:10), Whatman No. 3 paper impregnated with 10 % liquid paraffin in petroleum ether, (5) methanol–n-butanol–water (80:5:15), Whatman No. 3 paper impregnated with 10 % liquid paraffin in petroleum ether, (6) benzylalcohol, Whatman No. 3 paper impregnated with 20 % liquid paraffin in petroleum ether.

For the detection of various spots, the fluorescence of the dried chromatograms was first examined using a high pressure mercury vapour-lamp (Philips Philora HPW 125) equipped with a Wood glass filter. For the detection of steroids, antimony trichloride was generally used[13]. The dried chromatograms were immersed in a saturated solution of antimony trichloride in chloroform and dried for 5 min at 120°. For a more sensitive detection, chromatograms treated in this way were examined using an ultraviolet lamp as mentioned above. Occasionally iodine[14], phosphowolframic[15] and phosphomolybdenic acid[16], and blue tetrazolium[17] were used for the detection of spots.

For the detection of carbohydrates, spraying of the chromatograms with the benzidine reagent[18] was the method most used. Aniline hydrophthalate[19] and naphthoresorcine[20] were used in some instances.

Determination of nitrogen was carried out by MARKHAM's modification of the Kjeldahl method[21], determination of sugars by the orcinol method[22], using equimolar glucose and mannose mixture as a reference solution. Phosphorus was determined by the method of FISKE AND SUBBAROW[23] and cholesterol on the basis of the Liebermann–Burchhardt reaction[24]. Determinations of optical densities were performed in all instances in a Lange photoelectric colorimeter, using monochromatic metal interference filters.

Assay of biological activity

This was made by studying the effect of various preparations on the incorporation of labelled amino acids into the proteins of Ehrlich ascites carcinoma. All fractions were tested in triplicate. Each reaction mixture in a conventional Warburg flask contained 2 ml of Ehrlich ascites tumour suspension (diluted to contain 0.5 to 0.7 % protein with an isotonic KCl–$KHCO_3$ mixture), 0.1 ml of the labelled amino acid solution, and 0.1 ml of the appropriate dilution of the fraction to be tested or of the blank isotonic KCl–$KHCO_3$ mixture. 9 different doses of each preparation, varying from 1 mg to 10^{-8} mg, were usually tested for their effect on incorporation. In each run the control samples were incubated simultaneously with the experimental ones by shaking them in a Warburg apparatus at 37.5° for 45 min.

The method of preparing the protein samples after incubation as well as that used for the measurement of their specific radioactivity is given in detail in our recent paper[1].

Zero-time protein samples were always prepared in duplicate. The counts/min value of these zero-time samples did not exceed 10 % in the case of methionine and 4 % in the case of glycine, of the counts/min values of the incubated samples (these being usually 190–220 counts/min/mg of dried protein precipitate in the case of methionine, and 30–40 counts/min/mg of protein, in the case of glycine). Standard errors did not exceed ± 3 % for duplicate protein samples from each flask, and ± 5 % for average duplicate values of three identical reaction mixtures.

For the evaluation of activity of the various preparations, the mean specific radioactivity of protein sample was divided by that of a control protein sample obtained from a reaction mixture containing no tissue preparation (control). Only preparations, of which at least two neighbouring doses showed a definite stimulatory effect (at least 120 % of the control specimen's value) were said to be active. Fractions showing only a slightly inhibitory activity in the highest doses used or no effect on the incorporation at all dose levels were said to be ineffective.

EXPERIMENTAL

Preliminary fractionation of rat liver tissue

The active substance appeared to be rather stable. Freeze-dried liver retained its activity for several months. The activity of homogenates prepared from it did not diminish for at least 5 days at 20°. However, repeated slow freezing and thawing of the homogenate completely destroyed its activity. Freeze-drying left the activity unimpaired.

The active substance appeared to be present in the supernatant of a liver homogenate centrifuged at 20,000 *g* for 20 min at 18°. It did not dialyse and was not destroyed by trypsin. The activity was not affected by heating a homogenate at 70° for 60 min. On the basis of these facts, in particular the maintenance of the original activity after digestion or heating, it seemed very improbable that a protein would be responsible for the effect. On the other hand, the fact that the active principle became dialysable upon incubation with trypsin suggested that a low-molecular substance attached to a protein carrier might be involved in the effect. Therefore the possible involvement of protein-bound lipid was investigated.

For this purpose, freeze-dried rat liver was extracted in a Soxhlet apparatus with various solvents. In each case, 1 g of material was extracted with 200 ml of the solvent for a period sufficient to obtain an colourless fluid in the last portions of the extract. The reddish-brown total extract was evaporated to dryness under reduced pressure below 50°, the residue was homogenized in a glass homogenizer in isotonic $KCl-KHCO_3$ solution, and the resulting suspension, made up to the volume of a 1:10 homogenate of a corresponding amount of liver, was tested.

Benzene extract, ether extract, and acetone extract showed the same enhancing effect as the original homogenate. However, the ethanol extract was inactive. These results favoured our assumption that some lipid substance might be responsible for the activity.

Subfractionation of the unsaponifiable lipid fraction from varous tissues

It was found in some preliminary experiments that tissues (not only the tumours) from tumour-bearing animals seemed to contain a much higher quantity of the active substance than do the corresponding organs of normal animals. Therefore Walker tumour tissue and livers from rats bearing this tumour, as well as Sarcoma 37 tissue and livers from mice bearing this tumour, were used in these fractionation experiments.

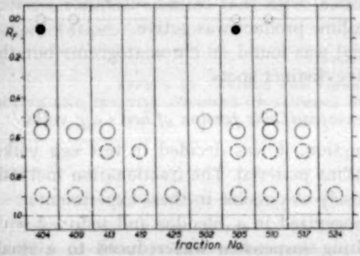
In each case, tissues were homogenized in ethanol in a blendor and refluxed with ethanolic potassium hydroxide. The resulting suspension was extracted with petroleum ether and this extract was then evaporated to dryness under reduced pressure. The dry residue was dissolved in petroleum ether and filtered. The solution obtained was sucked through an alumina column (Grade I). The column was eluted by a series of solvents and solvent mixtures (see Table I) of increasing polarity.

TABLE I

COMPOSITION OF VARIOUS SOLVENT MIXTURES USED FOR ELUTION OF ALUMINA COLUMNS

Composition	*Fraction No.*
Petroleum ether	1
Benzene (25 %) in petroleum ether	2
Benzene (50 %) in petroleum ether	3
Benzene (75 %) in petroleum ether	4
Benzene	5
Chloroform (25 %) in benzene	6
Chloroform (50 %) in benzene	7
Chloroform (75 %) in benzene	8
Chloroform	9
Acetone (25 %) in chloroform	10
Acetone (50 %) in chloroform	11
Acetone (75 %) in chloroform	12
Acetone	13
Ether (25 %) in acetone	14
Ether (50 %) in acetone	15
Ether (75 %) in acetone	16
Ether	17
Ethanol (25 %) in ether	18
Ethanol (50 %) in ether	19
Ethanol (75 %) in ether	20
Ethanol	21
Ethanol (75 %) in water	22
Ethanol (50 %) in water	23
Ethanol (25 %) in water	24
Water	25

In a typical experiment of this kind, 1000 g (wet weight) liver from Walker tumour rats was homogenized in 2000 ml 96 % ethanol in a blendor. 100 g potassium hydroxide were added to the homogenate, which was then refluxed for 4 h on a boiling water bath. After cooling and adding 200 ml water, extraction was carried out by shaking 10 times with a total volume of 30 l petroleum ether. The resulting brilliant yellow solution was evaporated to dryness under reduced pressure below 35°. 15 g of a reddish-brown dry residue was obtained. This was shaken with 500 ml petroleum ether, the resulting somewhat turbid solution filtered, and the clear, bluish-violet fluorescing filtrate poured on a column of 150 g alumina, activity I (diameter 25 mm, length 820 mm). After the solution had passed the column, the latter was successively washed with 500 ml each of the solvent mixtures given in Table I. After the last drop of a particular fluid had entered the top of the column, 500 ml of the next fluid was applied and a new fraction collected. Each fraction was evaporated to dryness under reduced pressure below 35°, the residue dissolved in a small volume of an appropriate solvent and tested by paper chromatography (Whatman paper No. 3, acetic acid–chloroform–liquid paraffin). Fractions giving corresponding chromatographic patterns were pooled. In this way the fractions designed by 605 (pooled fractions 1–5), 610 (6–10), 624 (11–24), and 625 (25) were obtained. Their chromatographic behaviour is illustrated by Fig. 2. These pooled fractions were evaporated to dryness, the residue of each was homogenized in isotonic $KCl-KHCO_3$ buffer in a glass homogenizer to give a 0.1 % suspension. These suspensions were tested.



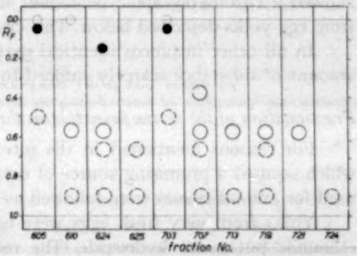


Fig. 1. Chromatographic pattern of unsaponifiable lipids of mice liver (fractions No. 404–425) and Sarcoma 37 tissue (fractions No. 502–524) in acetic acid–chloroform–liquid paraffin mixture. ●, Substances giving both steroid and sugar reactions; ———, steroid reactions only; ----, sugar reactions only; ······, compounds giving blue fluorescing spots on unsprayed paper and no other reactions.

Fig. 2. Chromatographic pattern of unsaponifiable lipids of rat liver (fractions No. 605–625) and Walker tumour tissue (fractions No. 703–724) in the same solvent mixture as in Fig. 1.

TABLE II

EFFECT OF FRACTIONS PREPARED FROM VARIOUS ANIMAL TISSUES ON THE INCORPORATION OF LABELLED METHIONINE INTO THE PROTEINS OF EHRLICH TUMOUR *in vitro*

*Fraction No.**	*Dose (10⁻ⁿ mg)*									*Activity*
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
605	123	62	70	96	75	80	132	220	215	+
610	69	182	252	99	96	63	132	110	137	+
624	117	169	152	75	60	95	153	123	112	+
625	85	93	87	59	85	78	71	74	70	—
703	65	128	79	131	91	108	72	97	102	+
707	68	97	72	79	69	81	90	68	59	—
713	96	54	81	228	163	190	139	134	128	+
719	63	83	90	69	84	76	129	125	92	+
721	34	38	116	92	49	128	114	82	83	+
724	89	89	95	123	132	110	93	104	101	+

All values given in this table are in percentage of control specimen's value.

* Fractions No. 605–625 are prepared from livers of tumour-bearing rats, fractions No. 703–724 from the Walker tumour tissue. The two last numbers denote the number of the pooled fraction (for instance fraction 605 = pooled fractions No. 1–5, 610 = pooled fractions No. 6–10, etc.).

In the corresponding fractionation of the petroleum ether extracts from ethanol–KOH digests of Walker tumours (680 g), Sarcoma 37 of mice (35 g), and liver of mice bearing this tumour (55 g), the volumes of elution fluids used were proportional to the amount of tissue.

Chromatographic patterns of various fractions from these tissues are given in Figs. 1 and 2, and their effect on the incorporation in Table II. Obviously most fractions are active.

Only in the case of rat liver tissue was it possible to recover a few milligrams of a crystalline active substance. For example, a portion of the residue of fraction 605 was dissolved in a small volume of ether and ethanol was added until a slight opalescence appeared. After a few days in an ice-box fine needle-shaped crystals appeared, which after two recrystallizations possessed a constant melting point of 65° and showed a chromatographic behaviour identical with that of the substance isolated from egg yolks described below. This crystalline product was active.

In all other instances identical material was found on chromatograms but the amount of substance scarcely sufficed to give distinct spots.

Fractionation of the active principle in the unsaponifiable portion of hen's egg yolks

For reasons mentioned in the introduction, it was decided to test egg yolks, which seemed a promising source of crystalline material. The fractionation methods used for animal tissues were followed as closely as possible in these experiments.

Yolks from very fresh eggs were homogenized in a blendor and refluxed with ethanolic potassium hydroxide. The resulting suspension was reduced to a small volume and extracted by benzene. The residue after extraction with benzene was tested for its activity in the usual way. It was found to be inactive, essentially all active material being present in the benzene layer. The benzene solution was evaporated and the residue dissolved in a small volume of petroleum ether. This was then poured on an alumina column.

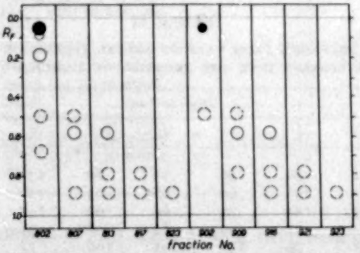


Fig. 3. Chromatographic pattern of unsaponifiable lipids of egg yolks fractionated on columns of inactive (fractions No. 802–823) and active alumina (fractions No. 902–923). The same solvent mixture was used as in Fig. 1.

Since preliminary experiments had suggested that some destruction of the active substance might occur on columns of highly active alumina, two columns, one of highly active grade I (the same as in fractionations of animal tissues) and another of weakly active grade IV alumina, were used in this experiment.

The petroleum ether solution obtained from 50 yolks was divided into two equal parts and poured on the two columns of alumina (diameter 25 mm, length 550 mm). They were then eluted with solvent mixtures as given in Table I (25 ml each). Again the chromatographically identical fractions were pooled and their activity tested. Chromatograms of these pooled fractions are given in Fig. 3.

Obviously, only small chromatographic dissimilarities exist between the fractions obtained from the two columns, while the similarity with the products from animal tissues is striking. On the other hand, very distinct differences do exist between the fractions obtained with both types of alumina as far as their effect on the incorporation is concerned (see Table III). While essentially all fractions of the grade I alumina column were active, only the first and the last fraction from the grade IV alumina proved to be effective. It seems very probable that a decomposition takes place on active alumina yielding substances, which are still active in amino acid incorporation.

TABLE III

EFFECT OF VARIOUS FRACTIONS PREPARED FROM EGG YOLKS ON ACTIVE AND INACTIVE COLUMNS OF ALUMINA ON THE INCORPORATION OF LABELLED METHIONINE INTO THE PROTEINS OF EHRLICH TUMOUR

*Fraction No.**	*Dose (10^{-8} mg)*									*Activity*
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
802	117	125	130	118	75	71	73	78	82	+
807	86	91	96	99	101	101	101	93	101	—
813	75	70	72	93	90	86	94	102	95	—
817	91	86	72	78	89	88	91	86	72	—
823	83	74	92	91	141	130	102	70	89	+
902	106	100	115	129	158	131	69	79	83	+
909	128	107	105	56	81	61	123	183	145	+
916	35	73	73	95	62	78	86	105	101	—
921	105	90	123	83	87	85	105	139	218	+
923	101	100	118	129	136	115	110	102	104	+

All values given in this table are in percentage of control specimen's value.

* Fractions No. 802–823 = inactive alumina (grade IV), fractions No. 902–923 = active alumina (grade I). Composition of each fraction is indicated by both last numbers as in Table II.

In an attempt to isolate the active substance in crystalline form, small portions of residues of fractions 802 and 902, respectively, were dissolved in ether, and ethanol was added until the solution became slightly opalescent. After a few hours in the ice-box a heavy crop of very fine needle-shaped crystals appeared. This crystalline substance had an identical melting point of 65° and an identical chromatographic behaviour in various systems as the crystalline substance isolated from rat liver.

Method of preparation of the active crystalline substance from egg yolks

The yolks from 50 fresh eggs were homogenized in a blendor and refluxed with 1000 ml 10% ethanolic potassium hydroxide for 4 h. After cooling, the fine greenish-brown suspension was concentrated at 40° under reduced pressure to a viscous dark green liquid (400 ml). This was extracted in several small portions by shaking with benzene. It is necessary to use a large excess of benzene (10–15 times the volume of the concentrated ethanol–KOH digest in each run) to obtain a good separation of the layers. The extraction was continued until the benzene layer remained uncoloured. Opalescent yellow benzene extracts (28 l) were pooled, centrifuged at 30,000 *g* in a Sharples centrifuge and the resulting brilliant yellow solution evaporated at 50° under reduced pressure. The dry, reddish-brown residue was dissolved in 400 ml 30% benzene in petroleum ether. The turbid dark brown solution was filtered and the resulting clear filtrate poured on a column prepared from 1000 g of alumina, grade IV (diameter 50 mm, length 460 mm). The column was eluted with 11 l 30% benzene in petroleum ether until the effluent solution gave no more brilliant bluish-violet fluorescence in ultraviolet light.

The eluate was evaporated at 40° under reduced pressure. A brown, oily residue was obtained, which solidified on standing at room temperature. This was dissolved in a small volume of ether, after which ethanol was added until the resulting solution appeared slightly opalescent. During the next 5 min the first very fine needle-shaped crystals appeared. Crystallization was completed at —30° overnight (lower temperatures were found preferable for higher yields of crystals). The crystals were spun down at 0° and washed several times with ethanol. The mother liquids were concentrated, and a second and a third crop of crystals obtained on standing at —30°. The crystals were pooled and after drying for 4 days in a high vacuum (0.05 mm Hg) 15 mg of a greasy, colourless substance was obtained.

After three recrystallizations from ethanol–ether the melting point was constant at 65°. The substance gave only one spot when chromatographed in five various solvent systems.

Some chemical properties of the crystalline substance

Elementary analyses showed the presence of carbon, hydrogen, oxygen, and phosphorus. At room temperature the substance is very soluble in petroleum ether, benzene, toluene, ether, chloroform, propanol, butanol, and cyclohexanol, but only very sparingly in acetone. It is also sparingly soluble in hot methanol and ethanol, and insoluble in cold or boiling water.

It gives colour reactions typical for steroids[25] (Liebermann–Burchhardt, Rosenheim, and Salkowski tests). It is not precipitated by digitonine. On paper chromatograms, it can be detected by iodine, phosphowolframic and phosphomolybdenic acid, blue tetrazolium, aniline hydrophthalate, naphthoresorcine, and in particular with benzidine. On papers sprayed with 10% trichloracetic acid and heated at 100° [26] it gives an intensively blue fluorescing spot. It also gives a blue fluorescence on unsprayed paper, but this is rather weak. It readily reacts with antimony trichloride to give an intensive reddish-pink spot on the paper.

In solution the substance gives some positive carbohydrate reactions, *e.g.* a deep red colour upon heating with phloroglucine, while the reactions with naphthoresorcine and orcine are also positive.

Paper chromatography is most conveniently carried out with solvent system (2). In water-saturated butanol the R_F value is extremely high (0.93), in acetic acid–chloroform–liquid paraffin, on the other hand, unsatisfactorily low (0.05). In the further systems tested, the active substance appeared not to move from the starting point at all. With the exception of water-saturated butanol all solvent systems mentioned on page 34 proved to be very efficient for detecting impurities in the crystalline preparations. For this reason they were all applied in our routine test for checking the purity of preparations.

The crystalline substance seems to be rather unstable. Upon prolonged heating or storing in solution for a long period of time it appears to decompose. It was found that its extinction coefficient at various wavelengths in the ultraviolet region is rapidly changing. It seems that, in particular, light and atmospheric oxygen are harmful. For this reason several steps of the isolation procedure, especially the crystallization, are now being performed under nitrogen. Upon standing in crystalline form the substance becomes yellowish brown and various products are formed, which can be distinguished from the original substance by paper chromatography.

The activity of the crystalline substance

Fig. 4 illustrates the enhancement as well as the inhibition of the incorporation of glycine and methionine in ascites protein by various doses of the substance. It is difficult to understand why two zones of inhibition are separated by a zone of activity. In particular the inhibition at low concentrations is obscure. It may be that the substance still contains an inhibitory factor whose action cannot be detected at higher concentrations of the stimulation factor, the latter also becoming inhibitory at still higher concentrations.

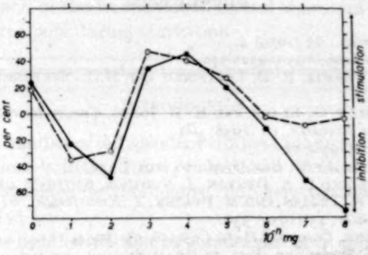


Fig. 4. Effect of the crystalline compound on the incorporation of labelled glycine (———) and methionine (– – – –) into the proteins of Ehrlich tumour *in vitro*. Individual points indicate averages of 5 experiments carried out with the same batch of Ehrlich ascites. Results of individual experiments did not differ more than ± 8%. Statistical treatment (*t*-test) of most values gave $P < 0.001$ when compared with the control.

It was shown above (see Table III) that some fractions isolated from yolks as well as animal tissues on active alumina have only the enhancing effect.

The inhibitory compound may be regarded as a destruction product of the active substance since similar compounds were also found in solutions of crystalline substance left for longer periods of time.

It is also possible that one substance is capable of influencing various enzymes resulting in various overall effects of different doses.

The values given in Fig. 4 are the results of experiments with one particular batch of Ehrlich ascites. With different batches, different absolute values for stimulation or inhibition were obtained. Nevertheless, the maximum stimulatory effect was always found at exactly the same dose. The stimulatory effect of the highest dose was always present; in some batches the 0.1 mg dose also had an enhancing effect.

DISCUSSION

The same substance seems to be present in such widely different materials as animal tissues and egg yolks. Further experiments[27] showed that it is capable of stimulating the growth of young animals very considerably when added to their diet. Therefore its presence as a growth-stimulating factor in relatively high amounts in egg yolks is understandable. The active substance was isolated only from fresh fertilized eggs. We are not yet quite sure if it is necessary for the eggs to have been fertilized. It is essential, however, that very fresh eggs are used.

The higher quantity of the active substance present in tissues of tumour-bearing animals as compared with those of normal ones suggests the relation of this compound to malignant growth.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. E. **Knobloch**, Research Institute for Pharmacy and Biochemistry, Prague, for his kindness in carrying out some analyses as well as for his valuable advice.

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TURNOVER OF PROTEIN AND NUCLEIC ACID IN SOLUBLE AND RIBOSOME FRACTIONS OF NON-GROWING *ESCHERICHIA COLI*

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(Received August 15th, 1959)

SUMMARY

The stability of protein and of RNA has been examined in the ribosome and soluble fractions of non-growing *E. coli*. In both fractions there is balanced degradation and re-synthesis of protein at about 5% per h. The RNA of the ribosomes is also degraded at this rate but re-synthesized at only 1.5% per h. In the soluble fraction the rate of nucleic acid synthesis exceeds the rate of degradation, so that there appears to be net transfer of material from the ribosomes to the soluble fraction. Degradation of ribosomes supplies much of the free amino acids and almost all of the ribonucleotides passing through the free pool during starvation.

INTRODUCTION

It has been shown in a number of independent studies that, in actively growing cultures of *Escherichia coli*, there is little, if any, breakdown of protein[1-4]. The stability of RNA has not been examined so thoroughly, but here too the evidence suggests a high degree of stability[4,5].

By contrast it has been found that in populations of *E. coli*, the growth of which has been prevented by nitrogen or carbon limitation, there is balanced degradation and resynthesis of protein which proceeds for some hours at about 5%/h[6-8]. This increased lability in the protein of stationary populations appears to be general among micro-organisms. In *Bacillus cereus* the rates of protein turnover in growing and non-growing cells are 1 % and 7 %/h respectively[9]. In yeast essentially similar results were obtained for breakdown of protein[10,11] and there were similar changes in the stability of the RNA. In these studies in bacteria and yeast the turnover was too great to be accounted for by death and lysis of the cells and it must therefore be presumed to be true intracellular turnover.

When individual enzymes have been measured, they have usually appeared to be stable under conditions where general protein turnover occurs. This appears to be true for nitratase and tetrathionase[12], lysine apo-decarboxylase[13] and β-galactosidase[7], and it raises the question whether turnover affects only non-enzymic proteins. It now appears that the active enzymes of the bacterial cell are distributed between the

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; TCA, trichloroacetic acid.

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soluble and cell membrane fractions and that the ribonucleoprotein particles (ribosomes) seem to be associated with very little enzymic activity[14]. It therefore seemed possible that the material for turnover is provided by the ribosome particles, which are drawn upon under conditions of carbon or nitrogen starvation to provide amino acids.

The present study had two objects: first to determine to what extent the soluble and ribosome fractions were involved in turnover, and second, whether turnover of RNA was co-ordinated with that of protein. The degradation of protein was measured by the loss of radioactive leucine from the soluble and ribosome portions of nitrogen-starved cells; resynthesis was measured by incorporation of labelled leucine into these fractions. The corresponding values for nucleic acid turnover were obtained by measuring incorporation or loss of labelled guanine.

METHODS

Organism: A strain of *E. coli* K12 requiring leucine and threonine was used. The cells were grown with aeration at 35° in minimal medium with 1% succinate as the carbon source and with the required amino acids[7].

Measurement of leucine incorporation

Bacteria were harvested in the exponential phase of growth (0.7–0.9 mg dry wt./ml) and starved by incubation for 20 min in the same medium with threonine omitted. The cells were then washed in phosphate buffer (0.05 *M*; pH 7.0), and suspended in the same buffer containing 1% succinate and incubated at a density of about 1 mg dry wt. cells/ml with DL-[1-^{14}C]leucine (500 μg/ml; specific activity about 10 counts/min/μg). Samples containing about 170 mg dry wt. of cells were taken at intervals and chloramphenicol added to give a final concentration of 25 μg/ml to stop further incorporation. The cells were centrifuged and washed in 40 ml tris-(hydroxymethyl) aminomethane (0.001 *M*; pH 7.4) containing magnesium acetate (0.01 *M*)[14]. They were suspended in 11 ml of the same solution and disrupted for 15 min in a Raytheon oscillator (10 kc). This treatment gave very reproducible yields of both ribosomal and soluble fractions. The suspension was centrifuged for 10 min at 10,000 rev./min to remove unbroken cells and debris and the supernatant was centrifuged at 40,000 rev./min (100,000 × *g*) for 1 h. The sedimented ribosomes were washed with 11 ml of Tris–Mg solution and the washings added to the supernatant fraction. The ribosomes were suspended in 5 ml of Tris–Mg solution and both fractions treated in the cold with an equal volume of 10% trichloroacetic acid (TCA). The precipitate was extracted with hot TCA (5%) and organic solvents and radioactivity measured as previously described[7]. The relative specific activity has been expressed as a percentage of the activity of completely labelled protein obtained from cells which had been grown from a small inoculum in the presence of labelled leucine.

Loss of labelled leucine from protein

Cells were grown and harvested as described above, but with [^{14}C]leucine in the growth medium. They were then starved, washed and incubated as before in the presence of DL-[^{12}C]leucine (500 μg/ml) to trap any labelled leucine that might be released by the degradation of protein. Samples were taken at the beginning and end of the incubation period and protein counted after isolation, as above, from the ribosomal and soluble fractions. Because of the difficulty of measuring accurately a small percentage loss in radioactivity, a sample was taken only after 4 h, when the expected loss would be about 20 %.

Incorporation of guanine

After growth and the same preliminary starvation and washing as before, cells were incubated in 1 % succinate-buffer with [8-^{14}C]guanine (50 μg/ml; specific activity 60–100 counts/min/μg). Ribosomal and soluble fractions were prepared and treated in the cold with TCA (final concentration 5 %). The precipitate was washed twice with ice-cold 5 % TCA, and then twice with acetone, ethanol, ethanol–ether and ether[7]. The residue consisting of nucleic acid and protein was weighed, and then extracted twice at 90° for 20 min with 7 ml TCA (5 %)[15]. The residue of protein was dried by washing with acetone and ether. Purines were isolated by the following modification of the method described by KERR *et al.*[16]. The combined TCA extracts were acidified with *N* H_2SO_4 (1 ml) and extracted three times by shaking with a double volume of ether to remove TCA. The volume of the aqueous layer was reduced to about 8 ml and the solution heated at 100° for 30 min to convert the purines to free bases which were then precipitated by the addition of 100 mg $AgNO_3$ in a volume of 0.5 ml. Tubes were left overnight in the cold and the precipitate washed twice with 0.1 *N* H_2SO_4 (5 ml). It was then extracted twice for 5 min at 100° with 0.1 *N* HCl (5 ml) and the combined extract evaporated to dryness *in vacuo*. The purines thus obtained were dissolved in 2 ml H_2O and a suitable amount (about 0.2 ml) placed on Whatman No. 3MM paper together with 100-μg amounts of the original labelled guanine used in the incubation medium. Chromatograms were developed for 36 h with isopropanol–HCl–H_2O (75:18:17)[17] and dried at room temperature. The guanine spots which were easily visible under a u.v. lamp, were cut out and extracted three times with H_2O (10 ml). "Blanks" of similar size were cut from the sheet and treated in the same way. The combined extracts were evaporated to a small volume and then made up to 5 ml in graduated tubes. The concentration of guanine was determined by the absorption at 249 and 290 mμ, and a suitable volume (usually 2 ml) plated in 3.5 cm^2 planchets for measurement of radioactivity. Correction for selfabsorption was unnecessary.

Loss of labelled guanine from nucleic acids

When cells growing in the usual synthetic medium had reached 0.3 mg dry wt./ml, [^{14}C]guanine (final concentration 2.5 μg/ml; specific activity about 100 counts/min/μg) was added, and the culture grown further to about 0.8 mg/ml. The cells were harvested and after the same preliminary treatment as in previous experiments, they were incubated in succinate-buffer with [^{12}C]guanine (50 μg/ml). At the beginning and end of the incubation period samples were taken for preparation of the ribosomal and soluble fractions. The procedure outlined above for the isolation of purines was then followed, except that guanine was not separated from adenine. The purines were extracted by HCl from the silver precipitate, taken to dryness, and counted.

RESULTS

Rates of degradation in soluble and microsomal protein

K12 (leu$^-$thr$^-$) was grown in 1 % succinate synthetic medium (400 ml) in the presence of DL-[1-^{14}C]leucine (300 μg/ml; specific activity 5 counts/min/μg). The cells were harvested at 0.8 mg dry wt./ml, starved and washed and incubated with shaking in 1 % succinate-buffer (350 ml) with unlabelled leucine for 4 h (see METHODS). Samples (175 ml) were taken at the beginning and end of this period. During the incubation the O.D. of the suspension increased 20 %. This is commonly found in coliform bacteria during nitrogen starvation and is probably due to formation of polysaccharides from succinate. The specific activity of the protein isolated from the soluble and microsomal cell fractions was measured.

The results of such an experiment appear in Table I. It will be seen that, while the total amount of protein in each fraction is virtually unchanged, there is a fall in the specific activity of both fractions: 25 % in 4 h in the soluble fraction and 21.5 % in the particulate. This difference between the two fractions is not significant, and in some experiments the rate of degradation of the particulate fraction was equal to, or slightly higher than, that of the soluble fraction. The values found are in good agreement with an overall degradation rate of about 5 %/h for coliform protein.

TABLE I

RATE OF PROTEIN DEGRADATION IN SOLUBLE(S) AND RIBOSOMAL (R) FRACTIONS OF NITROGEN-STARVED BACTERIA AS MEASURED BY THE LOSS OF LABELLED LEUCINE

E. coli previously grown in the presence of [^{14}C] leucine was incubated 4 h in succinate-buffer containing [^{12}C] leucine (500 μg/ml) (See text.) Radioactivity of protein isolated from the two fractions was measured.

Time (h)	*Fraction*	*Protein isolated (mg)*	*Counts/min/mg protein*	*% Counts lost*
0	S	32.0	286	—
	R	28.6	268	—
4	S	34.0	214	25
	R	29.4	210	21.5

Rates of synthesis of soluble and ribosomal proteins

Cells were grown in 600 ml medium supplemented with unlabelled leucine and threonine. They were harvested at a density of 0.75 mg/ml, starved, washed and incubated in 1 % succinate-buffer (525 ml) containing DL-[^{14}C]leucine (final concentration 500 μg/ml; specific activity 10 counts/min/μg). Samples were taken at 0, 120 and 240 min and treated with 4.5 ml chloramphenicol (1 mg/ml) to stop further incorporation. Protein was then isolated from the soluble and ribosome fractions and its radioactivity measured.

It was again found that the amount of protein in each fraction remained the same during the 4 h of nitrogen-starvation (Table II). Both fractions appeared to become labelled at a more or less linear rate. The specific activity of the protein has been calculated as a percentage of that of fully labelled protein, obtained by growing the cells in labelled leucine. On this basis the soluble proteins are 8 % labelled after 2 h and 16.5 after 4 h. The corresponding values for the particulate proteins were 10 and 21.5 %. The proteins of both fractions are thus being re-synthesized at a rate (4–5 %/h) corresponding fairly closely to their rate of degradation.

TABLE II

RATE OF PROTEIN SYNTHESIS IN SOLUBLE(S) AND RIBOSOMAL (R) FRACTIONS OF NITROGEN-STARVED BACTERIA

E. coli was incubated with [^{14}C]leucine (500 μg/ml) in succinate-buffer for 4 h (See text). The specific activity of protein isolated from the two cell fractions has been calculated as a percentage of the activity of fully labelled protein (608 counts/min/mg) isolated from cells grown in the presence of [^{14}C]leucine.

Time (h)	*Fraction*	*Protein isolated (mg)*	*Counts/min/mg protein*	*% labelling*
0	S	38.0	1	0
	R	24.7	1	0
2	S	39.2	48	8
	R	26.3	61	10
4	S	38.3	101	16.5
	R	24.2	129	21.5

Rates of degradation of soluble and ribosomal nucleic acids

Cells were grown as before in synthetic medium (400 ml). When the culture had reached a density of 0.34 mg/ml, the medium was supplemented with [8-^{14}C]guanine (final concentration 2.5 μg/ml; specific activity 100 counts/min/μg). The culture was harvested at 0.8 mg/ml and, after the usual preliminary treatment, the cells were incubated in 1 % succinate-buffer (350 ml) containing [^{12}C]guanine (50 μg/ml) as a "trap". Samples (175 ml) were taken at the beginning of the incubation and after 4 h, and the soluble and ribosomal fractions were separated and precipitated in the cold with TCA. The dried precipitates were weighed and extracted with hot 5 % TCA (See METHODS.) The nucleic acid in each fraction is taken to be the amount of material extracted in this step. The total radioactivity of the purine fraction was then measured.

Table III indicates that the weights of nucleic acid, like those of the protein remained fairly constant during the starvation period.

The particulate fraction lost 24 % of its radioactivity in 4 h. This rate compares closely with the loss of radioactivity from the protein of the same fraction and is of the same order as that previously reported for RNA turnover in non-growing *E. coli*[18].

TABLE III

RATE OF NUCLEIC ACID DEGRADATION IN SOLUBLE(S) AND RIBOSOMAL (R) FRACTIONS OF NITROGEN-STARVED BACTERIA AS MEASURED BY THE LOSS OF LABELLED GUANINE

E. coli previously grown in the presence of [^{14}C]guanine was incubated 4 h in succinate buffer containing [^{12}C]guanine (50 μg/ml). The total radioactivity present in the purines isolated from the two cell fractions was measured (see METHODS).

Time (h)	*Fraction*	*Total nucleic acid extracted (mg)*	*Total purine radioactivity counts/min*	*% counts lost*
0	S	9.4	13,960	—
	R	16.9	14,220	—
4	S	10.0	13,250	5
	R	16.3	10,780	24

By contrast the soluble fraction lost only 15 % of its labelled guanine in the same period. It should be emphasized, at this point that, while the ribosomal fraction contains only RNA, the soluble fraction contains both RNA and DNA. If the latter is stable and also the major component of this fraction, the actual rate of breakdown of the soluble RNA may be considerably higher than appears from the values in Table III.

Rates of synthesis of soluble and ribosomal nucleic acids

Cells were grown as before to a density of 0.9 mg/ml. After starvation and washing they were incubated in 1 % succinate-buffer (525 ml) containing [8-^{14}C]guanine (50 μg/ml; specific activity 66 counts/min/μg).

Samples (175 ml) were taken at 0, and 240 min, and the specific activity of the guanine was measured after its isolation from the soluble and ribosomal fractions.

TABLE IV

RATE OF NUCLEIC ACID SYNTHESIS IN SOLUBLE(S) AND RIBOSOMAL (R) FRACTION OF NITROGEN-STARVED BACTERIA

E. coli was incubated with [^{14}C]guanine (50 μg/ml) in succinate-buffer for 4 h. The specific activity of guanine isolated from the nucleic acids of the soluble and ribosomal fractions has been calculated as a percentage of the activity (66 counts/min/μg) of the guanine in the incubation medium.

Time (h)	*Fraction*	*Total nucleic acid extracted (mg)*	*Guanine isolated (μg)*	*Counts/min per μg guanine*	*% labelling*
0	S	10.8	30	0.7	1
	R	18.7	35	0.8	1
4	S	11.1	34	9.5	14.5
	R	17.6	40	4.0	6.0

In Table IV, the specific activity of the isolated guanine is expressed as a percentage of that of the guanine added to the incubation medium. The rates of nucleic acid synthesis, as measured by the degree of guanine labelling in the two fractions, differed considerably. The soluble fraction was 14.5 % labelled at the end of 4 h, while the ribosomal fraction was only 6.0 % labelled. This is in contrast to the rate of degradation in this fraction which was almost four times as high (compare Table III).

DISCUSSION

In *E. coli* in a non-growing state the proteins of the ribosomes and those of the soluble fraction appear to become equally labile and are both degraded at a rate of about 5 %/h. This process is balanced by a more or less equal rate of re-synthesis, so that the amount of protein in each fraction remains constant during a 4-h period in which the total turnover may exceed 20 %.

The nucleic acids present an entirely different picture. The nucleic acid of the ribosomes, which is virtually all RNA is degraded at the same rate as the protein. This suggests that if one of the components of the ribosome is attacked the other also becomes unstable and that the ribosome as a whole is broken down to amino acids and nucleotides. These results are consistent with the observation of TISSIÈRES AND

WATSON[14] that the ribosomes are far more resistant to the action of ribonuclease than purified ribonucleic acid. The rate of re-synthesis of the particulate RNA appears to be at a much lower rate viz. about 1.5 %/h, assuming that guanine incorporation is an accurate measure of the rate of synthesis. If this assumption is made, it follows that the ribosomes synthesized under conditions of nitrogen-deprivation have an abnormally high ratio of protein to RNA. The soluble nucleic acids behave in the opposite way during nitrogen-starvation. They are characterized by a negligible rate of degradation and a fairly high rate of synthesis. The net result would be a transfer of material from the particulate to the soluble state through the nucleotide pool.

It thus appears that when cells enter a state of starvation a balanced flow of amino acids and ribonucleotides to the free pool is established. Such states of starvation are likely to arise temporarily whenever bacteria growing in a complex medium reach a point where one substrate has been exhausted and some alternative is available which requires an induced enzyme. As a result of the turnover process a supply of amino acids becomes available for enzyme synthesis under these conditions. The kinetics of synthesis of β-galactosidase during starvation has been examined and it was found that there was no specific diversion of amino acids to this enzyme. Instead, the β-galactosidase constituted the same fraction of the total protein synthesized as it did in the growing cells[6].

It is currently believed that the ribonucleoprotein particles in mammalian cells are essential sites of protein synthesis, and it has been assumed that this is aiso their function in bacteria. The present findings indicate that they can, in addition, act as a reservoir which supplies almost half of the amino acids and virtually all of the ribonucleotides passing through the free pools during starvation.

ACKNOWLEDGEMENTS

We are indebted to Mrs. I. WINICOV for invaluable technical assistance.

This work was supported by U.S. Public Health Service (Grant No. 484-2598). One of us (J. M.) is indebted to the U.S. Educational Commission for a Travel grant.

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OBSERVATIONS RELATIVES AU ROLE DES PROTÉINES DU MILIEU DANS LES PHÉNOMÈNES DE TRANSFORMATIONS BACTÉRIENNES

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(Reçu le 15 juillet, 1959)

SUMMARY

Observations on the role of proteins contained in the medium in bacterial transformations

Ribonuclease interferes with bacterial transformations in at least three ways:

1. inhibition, attributed to its complexing with DNA;
2. appreciable delay in the establishment of bacterial absorptiveness for DNA molecules in the medium;
3. under conditions which exclude these two effects, the yield of transformed bacteria can be increased 2- or 3-fold in the presence of RNase.

Albumin has a role in bringing about bacterial absorptiveness, and besides it is assumed to prolong the duration of that condition.

INTRODUCTION

Lorsque nous avons entrepris ce travail nous nous sommes posé la question suivante: est-il possible, par l'addition de RNase d'interférer avec l'expression phénotypique d'un gène nouvellement acquis par transformation? Un résultat positif aurait pu fournir un système favorable à l'analyse du mécanisme par lequel l'acide ribonucléique intervient dans la synthèse des protéines (pour une revue récente, voir BRACHET[1]).

Notre but n'a pas été atteint. Nous avons, en revanche, observé que la ribonucléase —ainsi d'ailleurs que d'autres protéines —interfère avec les transformations du Pneumocoque à différents niveaux, clairement dissociables les uns des autres. L'intérêt de ces observations ne nous a pas paru justifier une étude approfondie, et nous les livrons ici telles qu'elles sont, c'est à dire très incomplètes. Incomplètes en particulier en ce sens que le degré de spécificité de l'action des protéines n'a pas été approfondi. Incomplètes aussi en ce sens que certaines expériences, pourtant évidentes n'ont pas été tentées: aussi certaines observations ont-elles dû être présentées de manière essentiellement descriptive, sans tentative d'interprétation quelque peu détaillée.

Les phénomènes observés, qui ont fait l'objet de deux notes préliminaires[2,3] peuvent être groupés comme suit: (a) Interaction de la ribonucléase avec l'ADN transformant: il s'agit vraisemblablement d'une complexion. (b) Effets de l'albumine

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Abréviations: ADN = acide désoxyribonucléique; RNase = ribonucléase.

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et de la ribonucléase sur l'établissement de la compétence*. (c) Effet favorable de protéines du milieu sur le rendement de la réaction entre les bactéries compétentes et l'ADN transformant. Cet effet peut être attribué soit à une prolongation de l'état de compétence des bactéries, soit à une augmentation de la réactivité des bactéries compétentes avec l'ADN. Le premier facteur pourrait être prédominant dans le cas de l'albumine et le second dans le cas de la ribonucléase.

MATÉRIEL ET MÉTHODES

Souches bactériennes

Le système de transformation utilisé dans ce travail est le transfert d'une haute résistance à la streptomycine à une souche de Pneumocoque sensible, par l'ADN d'une souche résistante[4]. La souche réceptrice utilisée, sensible à la streptomycine, est la souche R36A[5]. La souche donneuse, S^r-T55-1, a été obtenue par la transformation de R36A par l'ADN de la souche résistante RStr[4]. Les souches R36A et RStr nous ont été données par Madame H. EPHRUSSI-TAYLOR.

ADN transformant

L'ADN transformant a été isolé de la souche S^r-T55-1, essentiellement selon EPHRUSSI-TAYLOR[6]. La déprotéinisation a cependant été opérée par l'emploi de Duponol C[7], et le fractionnement au $MgSO_4$–alcool (qui élimine une fraction de l'acide ribonucléique) a été omis.

L'ADN transformant conserve parfaitement son activité en solution saline (NaCl 0.14 *M*) concentrée (environ 1 mg ADN/ml) à basse température (congelée ou non). Les dilutions sont stables dans l'extrait de levure à 1 % (EPHRUSSI-TAYLOR, communication personnelle) à basse température (congelées ou non).

Entretien des souches

Milieu NEL (EPHRUSSI-TAYLOR, communication personnelle) modifié par addition d'ions Ca^{++}: Bacto Neopeptone (Difco), 10 g; Extrait de levure (Difco) 2 g; NaCl 4,25 g; eau bidistillée 500 ml.

Ce milieu est amené à pH 8 par l'addition de NaOH; il est complété, au moment où le flacon est entamé, par l'addition de 0.001 volume de glucose à 25 %, 0.01 volume de $CaCl_2$ 0.1 *M*.

Les tubes de culture, utilisés pour l'entretien des souches (tubes à sang) contiennent 2.5 ml de milieu NEL complété et 0.05 ml de sang de cheval defibriné, et sont bouchés au coton cardé.

La conservation à long terme des souches est assurée par repiquage tous les mois et demi (homogénéisation de la culture précédente, dilution de cette culture dans un tube de milieu frais, croissance à 37° jusqu'à la phase stationnaire, qui correspond à la concentration d'environ $3 \cdot 10^8$ bactéries par ml. Ces tubes sont conservés à la glacière.

L'entretien à court terme des cultures est assuré par repiquage journalier (croissance de 3.5 h à 37°, prélèvement, sans homogénéisation préalable, de 0.1 ml pour ensemencer la culture suivante, qui est conservée à la glacière et incubée le lendemain).

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* Ce terme désigne l'état physiologique qui permet aux bactéries d'absorber des molécules d'ADN du milieu.

Dans ce milieu les Pneumocoques ont tendance à former des chaînes parfois extrêmement longues, alors que dans le milieu C (voir ci-dessus) on trouve surtout des diplocoques et des courts chapelets.

*Conditions d'établissement de la compétence**

Milieu C (Ephrussi-Taylor, communication personnelle) modifié par l'addition d'ions Ca^{++}: Casamino acids (pure ou certified) Difco, 10 g; NaCl, 8.5 g; eau bidistillée 1 l; NaOH 1 *N*, 5 ml.

Ce milieu est complété, chaque fois qu'un nouveau flacon est entamé, par l'addition de: 0.04 volume d'extrait de levure à 10 %, préalablement traité au charbon actif à pH 3 et stérilisé sur bougie[6], 0.01 volume de glucose 2.5 %, 0.01 volume de $CaCl_2$, 0.1 *M*.

Au moment de l'emploi, ce milieu complété est additionné de 0.05 volume de serumalbumine de boeuf (fraction V de Armour) à 40 mg/ml.

La culture journalière en tubes à sang est diluée, en général 10 fois, dans du milieu C (complété, y compris l'albumine) et incubée à 37° (culture de compétence).

Conditions de la transformation

La compétence atteint son maximum vers 60 min si la culture en tubes à sang a été diluée 10 fois dans le milieu C ("culture de compétence"). Les bactéries sont en général mises en présence de l'ADN transformant par dilution de la culture compétente dans du milieu frais** contenant l'ADN. Les conditions précises seront détaillées au fur et à mesure de l'exposé des expériences.

L'incubation est arrêtée, en principe, 75 à 90 min après la mise en contact des bactéries et de l'ADN. Ce temps suffit à assurer l'essentiel de l'expression phénotypique de la transformation, sans cependant que le nombre des bactéries transformées ait commencé à augmenter du fait de la multiplication cellulaire[4,8,9].

A ce moment une dilution appropriée de la culture est incorporée sur boîte de Pétri, dans du milieu gélosé à sang de cheval (Taylor[10]) contenant 50 mg/ml de streptomycine.

La technique qui vient d'être décrite (caractérisée par le fait que l'établissement de la compétence et la réaction des bactéries compétentes avec l'ADN sont dissociés) sera désignée pour des raisons de commodité sous le nom de "système dissocié". Dans certains cas nous avons aussi utilisé la technique qui était anciennement la plus employée. Dans ce système "non dissocié" l'ADN est présent dès l'ensemencement de la culture. Celle-ci est incubée suffisamment longtemps (généralement 4 à 5 h) pour permettre l'apparition de bactéries compétentes, leur réaction avec l'ADN et l'expression phénotypique de la transformation.

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* Ces milieux (sans addition de $CaCl_2$) ont été utilisés avec de bons résultats par Ephrussi-Taylor au cours d'un séjour aux Etats-Unis. Ils nous ont fourni, à Bruxelles, pendant près de deux ans, des taux de transformation élevés (1 à 10% de la population) de manière reproductible, à condition cependant d'ajouter des ions Ca^{++}, qui à la concentration optima (ajoutée) de 10^{-3} *M* améliorent le taux de transformation d'un facteur de l'ordre de 500 (La même amélioration a été obtenue en triplant la concentration d'extrait de levure.)

Ces mêmes milieux n'ont jamais donné de transformations satisfaisantes ni à H. Ephrussi-Taylor et Opara (à Paris) ni à R. Latarjet; ils ont cessé brusquement d'être utilisables entre nos mains. Il semble que cette variabilité doive être attribuée à un facteur de la peptone, labile et présent en quantité variable d'une préparation à l'autre.

** L'influence de la présence d'albumine sur le rendement, même après l'établissement de la compétence (voir p. 56–58), n'a été réalisé que tardivement. Aussi, sauf mention du contraire, le milieu frais dans lequel les bactéries compétentes sont diluées est du milieu C complété mais sans albumine.

Protéines

Les protéines utilisées sont ajoutées aux milieux de culture sous forme d'un petit volume de solution saline (NaCl 0.14 *M*) concentrée; un volume égal de la même solution saline, sans protéine, est ajoutée en même temps aux tubes témoins.

Ribonucléase: Armour, cristallisée. Dans d'autres expériences d'autres préparations commerciales (GBI, Worthington) ont été utilisées, ainsi que des fractions isolées par chromatographie (fraction "A") et mises obligeamment à notre disposition par L. LEDOUX. Lysozyme: Armour. Cytochrome *c*: Sigma. Albumine: serumalbumine de boeuf (fraction V de Armour) traîtée selon TAYLOR[10].

Mesure de la compétence par la technique de dilution

La compétence d'une culture peut être suivie en fonction du temps par la technique de dilution, déja utilisée antérieurement[9] et dont nous rappelons le principe.

Lorsque'une culture compétente est diluée dans du milieu frais, l'apparition de nouvelles bactéries compétentes est interrompue; si le milieu de dilution contient de l'ADN, les bactéries qui étaient compétentes au moment de la dilution pourront réagir avec cet ADN. Les nombres de transformations observés en fonction du temps de la dilution sont entre eux dans le même rapport, indépendamment de la concentration de l'ADN. On peut admettre qu'ils donnent une mesure fidèle de l'état de compétence de la culture.

Diluer la culture compétente dans du milieu frais revient à initier une nouvelle culture. Celle-ci développera une compétence "secondaire" au cours de la seconde moitié de sa croissance exponentielle, c'est à dire d'autant plus tard que la dilution est plus forte. Il est essentiel de calculer la dilution de sorte que la compétence "secondaire" soit suffisamment tardive, pour ne pas contribuer à la transformation. Si, pour une raison quelconque il n'est pas possible de diluer suffisamment, le même résultat est atteint par l'addition de DNase 15 à 20 min après la dilution. Les deux procédés concordent parfaitement.

Conformément à ce qui avait été observé précédemment dans d'autres milieux[9] la compétence apparaît d'autant plus tard que l'inoculum utilisé est plus faible. Elle occupe approximativement la seconde moitié de la phase de croissance exponentielle. La Fig. 2 (×) montre l'évolution de la compétence dans le système utilisé au cours de ce travail.

PARTIE EXPÉRIMENTALE

Interaction de la ribonucléase avec l'ADN transformant

En système "non dissocié" (voir p. 52) la présence de ribonucléase (Armour) entraîne une réduction massive du nombre des bactéries transformées. Une inhibition de 98 % a été observée pour 1000 µg/ml RNase et 2 µg/ml ADN. Ce type d'expérience ne permet pas de localiser l'effet de la ribonucléase. Signalons cependant qu'il ne peut être dû à une action toxique sur les cellules: la concentration de la ribonucléase la plus élevée que nous ayons utilisé (3000 µg/ml) n'a aucun effet défavorable sur la croissance. L'emploi de systèmes "dissociés" (voir p. 52) permet de localiser l'action de l'enzyme.

Dans un premier type d'expérience, tout effet éventuel sur l'établissement de la compétence est mis hors de cause: la culture de compétence ne contient ni ribonucléase, ni ADN, et ces substances n'interviennent qu'après une dilution des bactéries compétentes dans du milieu frais (dilution qui interrompt l'établissement de la compétence).

L'expérience dont les conditions et les résultats sont fournis par le Tableau I montre:

TABLEAU I

INHIBITION DE LA TRANSFORMATION PAR LA RIBONUCLÉASE

Des aliquotes d'une culture compétente (stabilisée par refroidissement à 0°) sont diluées 20 fois dans deux séries de tubes de milieu frais, contenant de l'ADN et de la RNase (Armour) aux concentrations indiquées dans le tableau. La température de la première série de tubes est de 37°; des dilutions de ces cultures sont coulées en gélose à la streptomycine après 75 min d'incubation, destinées à permettre l'expression du phénotype. La température de la seconde série de tubes est de 22°. Après 45 min à cette température, les cultures sont incubées 75 min à 37° pour compléter l'expression phénotypique; des dilutions sont alors coulées en gélose à la streptomycine Centres formateurs de colonies: $1.2 \cdot 10^8$/ml de culture compétente. Transformations par ml de culture compétente:

a. réaction à 37°	5 µg/ml ADN	$8.2 \cdot 10^5$
	0.05 µg/ml ADN	$3.3 \cdot 10^4$
b. réaction à 22°	5 µg/ml ADN	$1.6 \cdot 10^6$
	0.05 µg/ml ADN	$1.1 \cdot 10^4$

Concentration de la RNase	*200 µg/ml*	*500 µg/ml*	*1000 µg/ml*
	% d'inhibition de la transformation		
(*a*) *réaction à 37°*			
5 µg/ml ADN	75.6	$96._8$	$99._4$
0.05 µg/ml ADN	99.9_4	99.9_7	99.9_4
(*b*) *réaction à 22°*			
5 µg/ml ADN	$89._3$	$99._0$	$99._3$
0.05 µg/ml ADN	99.9_8	99.9_8	99.9_6

1. Que la ribonucléase peut provoquer une réduction massive de la transformation en agissant sur un stade postérieur à l'établissement de la compétence.

2. Que le pourcentage d'inhibition de la transformation dépend non seulement de la concentration de la ribonucléase, mais aussi, de façon très marquée, de la concentration de l'ADN transformant: une même concentration de ribonucléase est d'autant plus active que la concentration de l'ADN transformant est plus faible.

S'il s'agissait d'une action de la ribonucléase sur les cellules, on s'attendrait à ce que l'effet dépende essentiellement de la concentration de la ribonucléase. Que l'effet dépende très largement de la concentration en ADN suggère une interaction RNase-ADN (complexe?).

3. La comparaison des deux parties de l'expérience (où la réaction des bactéries compétentes se produit, respectivement, à 37° et à 22°) montre que l'effet est au moins aussi prononcé à 22° qu'à 37°. Ceci suggère que l'activité enzymatique de la ribonucléase —dont le coéfficient de température est de l'ordre de 5 —ne joue aucun rôle dans l'effet observé.

L'expérience suivante est destinée à voir si la ribonucléase du milieu peut encore produire son effet inhibiteur lorsque la réaction entre les bactéries compétentes et l'ADN libre du milieu a été pratiquement bloquée par dilution. La Fig. 1 montre que ce n'est pas le cas. Ce résultat renforce — sans la prouver —l'hypothèse selon laquelle l'effet inhibiteur de la ribonucléase résulte d'une interaction (complexe?) avec l'ADN libre du milieu.

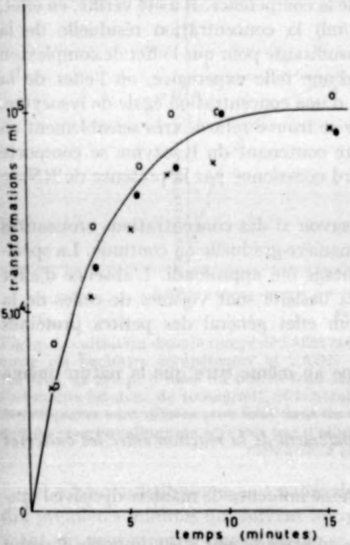

Fig. 1. Absence d'effet inhibiteur de la ribonucléase après l'interruption de la réaction des bactéries compétentes avec l'ADN libre du milieu. Les bactéries compétentes sont diluées (1 : 1) au temps 0 dans du milieu frais contenant 5 µg/ml ADN. Aux temps indiqués en abscisses, la réaction des bactéries compétentes avec l'ADN libre du milieu est pratiquement interrompue par dilution (100 fois) dans du milieu frais contenant 0 (×), 500 (○) ou 3000 (●) µg/ml RNase (Armour). En ordonnées, les nombres de transformations, rapportés à 1 ml de culture compétente non diluée.

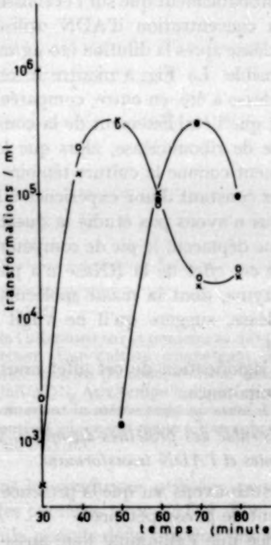

Fig. 2. Effet de la ribonucléase sur l'établissement de la compétence. La compétence a été suivie en fonction du temps, en utilisant la technique de dilution, dans trois cultures parallèles. La première (×) est une culture témoin, la seconde (○) contient 2 mg/ml lysozyme et la troisième contient 2 mg/ml RNase (Armour).

Effets de l'albumine et de la ribonucléase sur l'établissement de la compétence

Le succès des transformations du Pneumocoque dépend, entre autres, de la présence d'un facteur que HOTCHKISS ET EPHRUSSI-TAYLOR[11] ont pu identifier à l'albumine; les milieux utilisés pour les transformations de Pneumocoque sont toujours additionnés de serum ou d'albumine purifiée. Nous avons observé précédemment[9] que l'effet principal de l'albumine porte sur l'établissement de la compétence: si l'albumine est ajoutée à la culture de compétence avec un retard croissant l'ordre de compétence apparaît du plus en plus tard.

L'effet de la ribonucléase sur l'établissement de la compétence a été étudié de la manière suivante: Des cultures de compétence sont ensemencées simultanément;

le milieu de culture a été additionné préalablement de 0·1 volume de solution saline (NaCl 0.14 M) contenant ou non 20 mg/ml RNase (Armour). Après différents temps d'incubation, la compétence est déterminée par dilution (100 fois) dans du milieu frais contenant de l'albumine (2000 μg/ml) et 5 μg/ml ADN, incubation de 75 min à 37° et étalement en gélose à la streptomycine. Dans ces conditions la ribonucléase n'agit probablement que sur l'établissement de la compétence. Il a été vérifié, en effet, qu'à la concentration d'ADN utilisé (5 μg/ml) la concentration résiduelle de la ribonucléase après la dilution (20 μg/ml) est insuffisante pour que l'effet de complexion soit sensible. La Fig. 2 montre le résultat d'une telle expérience, où l'effet de la ribonucléase a éte, en outre, comparée à celui d'une concentration égale de lysozyme. On voit que l'établissement de la compétence se trouve retardé très sensiblement en présence de ribonucléase, alors que la culture contenant du lysozyme se comporte exactement comme la culture témoin; le retard occasionné par la présence de RNase est assez constant d'une expérience à l'autre.

Nous n'avons pas étudié la question de savoir si des concentrations croissantes d'enzyme déplacent le pic de compétence de manière graduelle ou continue. La spécificité de cet effet de la RNase n'a pas davantage été approfondi. L'absence d'effet du lysozyme, dont la masse moléculaire et la basicité sont voisines de celles de la ribonucléase, suggère qu'il ne s'agit pas d'un effet général des petites protéines basiques.

La signification de cet effet nous échappe au même titre que la nature même de la compétence.

Effet favorable des protéines du milieu sur le rendement de la réaction entre les bactéries compétentes et l'ADN transformant.

1. Nous avons vu que la présence d'albumine influence de manière décisive l'établissement de la compétence.

L'idée que l'albumine joue aussi un rôle ultérieurement s'est imposée lorsque nous avons réalisé qu'une assez forte dilution des bactéries dans du milieu frais sans albumine peut réduire d'un facteur de plusieures unités le rendement de la transformation. Ce résultat est imputable, plutôt qu'à la dilution elle-même, à une concentration résiduelle trop faible de l'albumine. La question de savoir si cet effet est spécifique de l'albumine ou s'étend au contraire à d'autres protéines ou, plus généralement, à d'autres macromolécules n'a pas été étudiée. Un effet favorable de la ribonucléase se manifeste dans des conditions où les deux effets inhibiteurs sont éliminés, mais le mécanisme de cet effet pourraît être différent de celui de l'albumine.

Un premier type d'expériences était destiné à localiser dans le temps l'effet de l'albumine. Une premiere dilution, dans du milieu contenant de l'ADN, interrompt l'établissement de la compétence et déclenche en même temps la réaction des bactéries avec l'ADN. La seconde dilution a lieu à différents temps dans du milieu contenant la même concentration d'ADN et une concentration finale d'albumine, soit de 2000 μg/ml, soit de 2 μg/ml. Les résultats d'une telle expérience sont représentés par la Fig. 3. On voit qu'une seconde dilution à différents temps, dans du milieu contenant la même concentration d'ADN et une concentration élevée d'albumine n'a pas d'effet sensible sur la transformation, quel que soit le moment de cette dilution. Au contraire, aussitôt après la première dilution, une seconde dilution dans du milieu contenant toujours la même concentration d'ADN mais une concentration finale d'albumine de

2 μg/ml seulement, réduit le nombre de transformation d'un facteur 2; si cette seconde dilution est différée, la réduction du nombre des transformations diminue pour disparaître à partir d'un intervalle de 10 min entre les deux dilutions. Les paliers des deux courbes sont égaux.

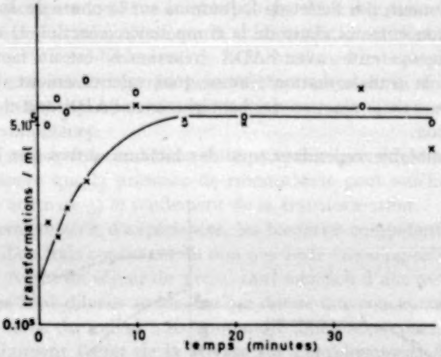

Fig. 3. Localisation dans le temps de l'effet favorable de l'albumine sur le rendement de la réaction entre les bactéries compétentes et l'ADN transformant. Une culture compétente est diluée (10 fois) au temps 0 dans du milieu frais sans albumine ajoutée (la concentration résiduelle de l'albumine est donc de 200 μg/ml), et contenant 5 μg/ml ADN. Aux temps indiqués en abscisses, des aliquotes sont diluées (100 fois) dans du milieu contenant la même concentration d'ADN et, soit 2000 μg/ml albumine (○) soit pas d'albumine ajoutée (×), ce qui mène à une concentration résiduelle d'albumine de 2 μg/ml.

Le besoin d'albumine se localise donc, dans les conditions utilisées, pendant les dix premières minutes qui suivent le contact des bactéries avec l'ADN. Plus précisément, on peut montrer que ce besoin se limite à la période de la réaction des bactéries compétentes avec l'ADN libre du milieu: si cette réaction est bloquée par une forte dilution dans du milieu sans ADN, la présence ou l'absence d'albumine dans le milieu de dilution n'influence pas le nombre des transformations (Fig. 4).

Il paraît donc raisonnable d'envisager un effet de l'albumine soit sur la réactivité des bactéries compétentes avec l'ADN soit sur le maintien de l'état de compétence, les deux possibilités ne s'excluant du reste pas. Si la seconde éventualité intervient, la concentration de l'albumine dans le milieu de dilution doit influencer le résultat de la transformation même si l'ADN n'est pas encore présent. L'expérience suivante, décrite par la Fig. 5, montre qu'il en est bien ainsi.

Les trois courbes de la Fig. 5 doivent être comparées deux à deux. Les conditions qui fournissent les courbes A' et B ne diffèrent qu'en ce qui concerne la concentration de l'albumine avant l'addition de l'ADN transformant; en revanche les conditions de la réaction entre les bactéries compétentes et l'ADN sont identiques. L'examen de ces deux courbes permet donc de comparer l'allure de la chute de compétence dans des milieux contenant respectivement 2 μg/ml et 2000 μg/ml d'albumine. On voit qu'en présence d'une forte concentration d'albumine, la chute de la compétence est nettement ralentie.

Les conditions des courbes A et A' ne diffèrent qu'en ce qui concerne la concentration de l'albumine, à partir du moment de l'addition de l'ADN. Chacune de ces deux courbes donne une image de la chute de compétence dans le milieu contenant 2 µg/ml d'albumine; comme on doit s'y attendre, la décroissance est parallèle. Les nombres absolus de transformations diffèrent, de la courbe A à la courbe A', d'un facteur qui représente l'effet de l'albumine après l'addition d'ADN. Ce point peut être expliqué, du moins partiellement, par l'effet de l'albumine sur la chute de la compétence. En effet, la compétition entre la chute de la compétence (réaction 1) et la réaction des bactéries encore compétentes avec l'ADN (réaction 2) est un facteur déterminant du rendement de la transformation[9]; aussi, tout ralentissement de la chute de la compétence au cours de la réaction des bactéries avec l'ADN doit-il améliorer le taux de la transformation.

Il est vraisemblable cependant, que des facteurs autres que le maintien de la

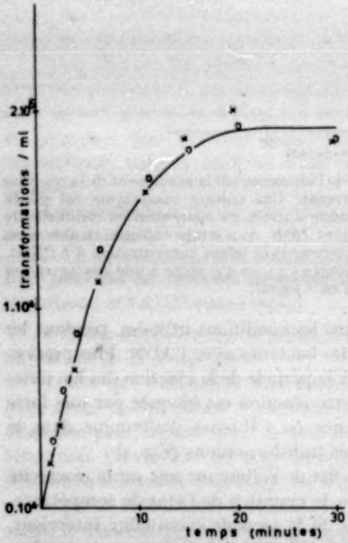

Fig. 4. Absence d'effet favorable de l'albumine après l'interruption de la réaction des bactéries compétentes avec l'ADN libre du milieu. Une culture compétente est diluée 100 fois (temps 0) dans du milieu sans albumine ajoutée, contenant 5 µg/ml ADN. Aux temps indiqués en abscisses la réaction des bactéries compétentes avec l'ADN libre du milieu est interrompue par dilution (100 fois) dans du milieu sans ADN, contenant soit 2000 µg/ml albumine (○), soit pas d'albumine ajoutée (×), ce qui donne une concentration residuelle d'albumine de 2 µg/ml.

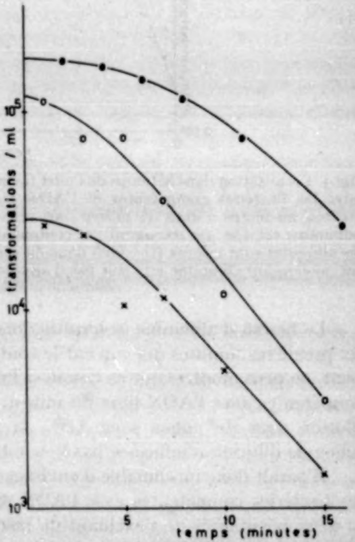

Fig. 5. Effets de l'albumine sur la chute de la compétence après dilution et sur la réaction des bactéries compétentes avec l'ADN. Une culture compétente est diluée 1000 fois (1) dans du milieu sans albumine (concentration résiduelle en albumine: 2 µg/ml), (2) dans du milieu contenant 2000 µg/ml albumine. La chute de la compétence dans les deux cultures diluées est suivie en fonction du temps par transfert d'aliquotes dans des tubes contenant l'ADN transformant (concentration finale: 0.5 µg/ml). Les trois courbes correspondent respectivement aux conditions suivantes: A (×) aliquotes de la dilution (1), transférées dans des tubes ne contenant que l'ADN; A' (○) aliquotes de la dilution (1), transférées dans des tubes contenant, outre l'ADN, suffisamment d'albumine pour que la réaction des bactéries compétentes avec l'ADN ait lieu en présence de 2000 µg/ml albumine; B (●) aliquotes de la dilution (2), transférées dans des tubes contenant l'ADN.

compétence, puissent contribuer à l'effet favorable de l'albumine. Si l'albumine n'agissait que sur la compétition entre les réactions 1 et 2 on s'attendrait, en effet, à ce que l'effet disparaisse aux concentrations saturantes d'ADN, où la vitesse de la réaction 2 est suffisante pour que tous les sites compétents aient eu le temps de réagir avant de perdre la compétence. Il ne semble pas que tel soit le cas.

2. Deux effets de la ribonucléase sur la transformation ont été décrits plus haut. Le premier résulte vraisemblablement de la formation d'un complexe ADN-RNase et se traduit par une inhibition, qui peut être complète de la transformation. Le second, dont la signification nous échappe totalement, consiste en un retard de l'établissement de la compétence.

Lorsque l'on utilise des conditions telles que ces deux effets soient éliminés ou minimisés, on observe que la présence de ribonucléase peut améliorer sensiblement (d'un facteur de l'ordre de 3) le rendement de la transformation.

Dans une première série d'expériences, les bactéries compétentes ont été diluées 10 fois dans du milieu frais contenant ou non une forte (2000 μg/ml) concentration de RNase (Armour). Après un séjour de 3 min, sauf mention d'une autre durée, dans ce milieu, les cultures sont diluées 40 fois (ce qui donne une concentration résiduelle de 50 μg/ml RNase) dans du milieu frais contenant différentes concentrations d'ADN. Ces conditions éliminent l'effet de la RNase sur l'établissement de la compétence. D'autre part, on peut s'attendre, d'après les résultats représentés par le Tableau I, à ce que la concentration résiduelle de RNase (50 μg/ml) soit ou non, selon la concentration de l'ADN, suffisante pour produire une complexion appréciable. Effectivement, aux concentrations faibles d'ADN (0.5 ou 0.05 μg/ml) l'inhibition de la transformation est nette; à la concentration de 5 μg/ml d'ADN l'effet favorable de la ribonucléase se manifeste. Dans certains cas la durée du traitement à la ribonucléase et le temps choisi pour interrompre par dilution l'établissement de la compétence ont été variés, sans effet sur l'amélioration du taux de transformation par la ribonucléase. Il paraît clair, en particulier, que la chute de la compétence n'est pas retardée par la présence de RNase. Le palier de la courbe donnant la fréquence de la transformation en fonction de la concentration d'ADN est le même, que les bactéries compétentes aient été ou non traitées par la ribonucléase. Ceci suggère que l'effet de la RNase, n'est pas d'augmenter le nombre des bactéries transformables mais d'accroître la vitesse de leur réaction avec l'ADN.

Dans des conditions où l'effet favorable n'est pas intentionnellement dissocié de l'effet de complexion on peut observer, selon les concentrations de RNase et d'ADN, toute la gradation depuis une forte inhibition jusqu'à un accroissement sensible du taux de transformation. L'effet de complexion domine aux fortes concentrations de ribonucléase, de l'ordre de 100 μg/ml. Aux concentrations plus faibles de RNase l'effet favorable peut l'emporter si la concentration de l'ADN est appropriée. Ajoutons que différentes préparations commerciales de ribonucléase se comportent différemment en ce qui concerne l'effet complexant ou, au contraire, activateur, d'une concentration donnée.

DISCUSSION

1. L'un des effets de la ribonucléase sur les transformations du Pneumocoque se traduit par une inhibition, qui peut être quasi-totale, du phénomène. Les expériences

décrites sont compatibles avec l'idée que cet effet résulte de la formation de complexes entre l'ADN et la RNase. La formation de tels complexes a été décrite à diverses reprises. Citons la démonstration par MAC DONALD (communication personnelle) d'une inhibition de l'activité enzymatique de la RNase par l'ADN. Dans ce cas, le constituant susceptible d'un dosage d'activité biologique est la ribonucléase et l'agent inhibiteur est l'ADN, de sorte que la situation est, en quelque sorte, symétrique de celle qui est décrite dans le présent travail. Un complexe RNase–ADN a aussi été mis en évidence par électrophorèse[12]. On peut imaginer que ce type de complexes est peu spécifique, et semblable à celui que l'ADN formerait avec n'importe quelle protéine basique; le présent travail ne contient, cependant, aucun renseignement à cet égard. Rappelons que l'acide apurinique (produit de la dégradation acide ménagée de l'ADN) est rendu acidosoluble non seulement par la RNase[13] mais aussi par d'autres protéines basiques[14].

On peut se demander par quel mécanisme la formation d'un complexe entre l'ADN transformant et la RNase empêche l'établissement de la transformation. Une agrégation des molécules d'ADN aurait pour conséquence une diminution du nombre de particules transformantes et, vraisemblablement, des difficultés accrues de pénétration de l'agent transformant dans les bactéries. Il n'est pas exclus, cependant, que des molécules d'ADN, même combinées à une ou plusieurs molécules de ribonucléase, puissent néanmoins pénétrer dans les bactéries; dans ce cas, on peut concevoir que la transformation échoue parce que la RNase, introduite dans la cellule par ce procédé du type "cheval de Troie", interrompt l'une des étapes de la transformation ou, plus brutalement, tue la cellule*. Il a été montré dans une série de cas que la RNase peut, effectivement, tuer les cellules dans lesquelles elle réussit à pénétrer (revue BRACHET[16]).

2. L'effet favorable qui peut être mis en évidence dans certaines conditions peut probablement être attribué, lui aussi, à la nature basique de la RNase. Ainsi que nous l'avons vu plus haut, tout se passe comme si la réaction entre l'ADN et les bactéries compétentes traitées à la RNase était accélérée. La réduction de la charge nette négative des cellules pourrait intervenir. Une interprétation plus précise de ce phénomène sera dans doute fournie par le développement des observations récentes selon lesquelles diverses substances basiques, telles que les polyamines trouvées dans les bactériophages, et diverses protéines basiques, peuvent dans certaines conditions améliorer le rendement des transformations (GOODGAL, communication personnelle; MARMUR, communication personnelle).

3. Nous n'avons aucune indication en ce qui concerne la signification du retard de l'établissement de la compétence en présence de RNase. Rappelons que le lysozyme, dont la masse moléculaire et la basicité sont voisines de celles de la RNase, est sans effet.

4. En ce qui concerne l'effet de l'albumine soit sur l'établissement de la compétence, soit aux stades ultérieurs, on peut faire deux remarques: (a) L'idée que les protoplastes bactériens seraient capables d'absorber de l'ADN du milieu dans des conditions où les cellules normales ne peuvent le faire —en d'autres termes, que la

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* Remarquons qu'un tel mécanisme pourrait concilier deux points de vue en apparence contradictoires: (a) la démonstration par LERMAN ET TOLMACH[15] de la spécifidité de l'état de compétence vis-à-vis de l'ADN, à l'exclusion de l'ARN (et, *a fortiori*, des protéines). (b) La suggestion[9] selon laquelle la DNase pourrait bien pénétrer dans les cellules compétentes. Selon le mécanisme du "cheval de Troie", la DNase pénétrerait dans les cellules compétentes à la faveur de leur fixation sur une molécule d'ADN.

paroi cellulaire est l'obstacle majeur de la transformation —doit être très répandue, si l'on en juge par le nombre de travaux en cours, tendant à transformer des protoplastes ou à les infecter avec des préparations d'ADN de phage. (b) L'albumine est un facteur de stabilité des protoplastes, ou du moins de certaines préparations[17, 18]. Il serait absurde d'assimiler les bactéries compétentes à des protoplastes, car aucune tendance à la formation de telles structures ne s'observe au microscope lors de l'établissement de la compétence. On peut néanmoins se demander si certaines propriétés de la surface des bactéries compétentes ne pourraient pas être, ne fût-ce que localement, voisines de celles des protoplastes. Deux observations, l'une favorable, l'autre défavorable à cette idée, peuvent être citées ici: (a) Il existe une certaine corrélation entre la chute de viabilité d'une population de Pneumocoque soumise au choc osmotique et son degré de compétence. (b) Lorsqu'une culture compétente est fortement diluée dans du milieu frais le titre viable n'est pas différent selon que ce milieu contient ou non de l'albumine (Thomas, observations non publiées).

RÉSUMÉ

La ribonucléase interfère, à trois niveaux au moins, avec les transformations bactériennes. On observe:

1. Un effet inhibiteur, dû vraisemblablement à la formation d'un complexe entre l'acide désoxyribonucléique et la ribonucléase;
2. Un délai sensible de l'établissement de la compétence;
3. Dans des conditions qui excluent les deux effets précités, le rendement de la transformation peut être amélioré d'un facteur 2 ou 3 en présence de ribonucléase.

L'albumine joue un rôle dans l'établissement de l'état de compétence, et intervient également à un second niveau, vraisemblablement en prolongeant la durée de la compétence.

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THE APPLICABILITY OF FORMOL TITRATION TO THE PROBLEM OF END-GROUP DETERMINATIONS IN POLYNUCLEOTIDES. A PRELIMINARY INVESTIGATION

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(Received July 29th, 1959)

SUMMARY

The reaction between cytidine-5′-phosphate and formaldehyde has been investigated in order to determine whether or not titration in the presence of added formaldehyde can be used as a means of measuring the length of polynucleotide chains. In 2 *M* formaldehyde the reaction with the nucleotide comes to equilibrium within 40 min at room temperature. The extent to which the pK_a of the cytidylic acid amino group is lowered by increasing formaldehyde concentrations has been measured. In potentiometric titration of cytidine-5′-phosphate in the presence of formaldehyde, good agreement was obtained between the amount of phosphate calculated from the titration curve and that determined by phosphorus analysis. When four molar equivalents of cytidine were also present, however, (a mixture approximately equivalent to a solution of a penta-cytidylic acid), the best estimate of phosphate made from the titration curve exceeded the true value by about 19%.

INTRODUCTION

A frequently recurring problem in polynucleotide and nucleic acid chemistry is that of determining the number of chain ends in a sample. It is generally accepted that many of the ends will carry singly-esterified phosphoric acid residues, and attempts have been made to estimate the number of such groups either by determining the amount of inorganic phosphate liberated by a purified phosphatase preparation or by analyzing the titration curves of the undegraded polymers. The enzymic approach has the disadvantage that the specificity of the phosphomonoesterase employed may not permit it to remove all of the terminal phosphoric acid residues. At the same time, the titration curves of polynucleotides represent the summation of many different dissociations, and it is necessary to assign dissociation constants to the various groups before an estimate of the number of terminal phosphate residues can be made. It is almost certain that values of the constants for the various types of ionizing group in a polynucleotide differ from those for similar groups in the mononucleotides. In particular, possibilities for hydrogen-bonding and neighboring-group charge effects are likely to raise the pK_a values of the amino groups of the nucleotide residues within a polynucleotide chain to the point where they will be mistaken for secondary phosphate groups[1]. Interference of this type is to be most expected from the cytidylic (or deoxycytidylic) acid residues, since the pK_a's of the amino groups of these nucleotides are appreciably higher than those of the corresponding groups of adenine or guanine nucleotides. Such interference is likely to be particularly serious in polynucleotides of biological interest, where the ratio of secondary phosphoryl dissociations to interfering amino group dissociations is almost certain to be small.

The classical formol titration of Sørensen has been used in the fields of amino acid and protein study for many years. In this procedure formaldehyde in aqueous solution is used to lower the dissociation constants of the amino groups of α-amino acids (or of protein side-chains) so that they titrate in a pH range where a conventional indicator can be used[2]. It seemed plausible, therefore, that formaldehyde could also be used to lower the pK_a values of the amino groups of nucleotides to the extent necessary to "uncover" the dissociations of the terminal phosphoric acid residues. Nucleosides and nucleotides that have amino groups are known to give a reaction with formaldehyde that can be detected spectrophotometrically[3]. It is thus possible to verify, in any instance, the completion of the reaction between the amino groups of a nucleotide and formaldehyde. The alteration of the titration curve of polyadenylic acid by formaldehyde, ascribed to the lowering of the pK_a values of the amino groups, has been reported[1]. The titration of a nucleic acid preparation in the presence of formaldehyde is also mentioned in the literature[4], but as yet no systematic study of the possible utility of formaldehyde for the determination of end-groups in polynucleotides has been made. The present paper is a report of an investigation of the behavior of the dissociation of the amino group of cytidylic acid in the presence of formaldehyde.

MATERIALS AND METHODS

Cytidine-5'-phosphate was obtained from Pabst Laboratories, Milwaukee, Wisconsin.

Cytidine sulfate monohydrate came from the California Corporation for Biochemical Research, Los Angeles.

Inorganic salts were reagent grade.

Formaldehyde was the reagent-grade 36–38 % aqueous solution containing 10–15 % methanol preservative. Prior to use it was passed through a column of Permutit CCG anion-exchange resin (Permutit Company, 330 W. 42nd St., New York, N. Y.), which lowered the formic acid concentration from 0.013 % to a negligibly small value. The resin treatment was found to introduce detectable buffering at about pH 6 in the effluent, probably through the introduction of bicarbonate ion, but the amount so introduced could be kept small by the use of 1 *M* ammonium hydroxide to regenerate the column. In titration experiments correction was made for this contamination by means of a control titration. Formaldehyde solutions treated with the resin in the manner described showed virtually no accumulation of formic acid after eight days storage at room temperature, nor could any accumulation be detected after incubation at pH 8.5 for up to 4 h at 37°. Following removal of the formic acid the molarity of each formaldehyde solution was calculated from its formaldehyde content, as determined by titration in the presence of sodium sulfite[5].

Formaldehyde concentrations in dilute solutions were calculated by assuming in each case that the volumes of concentrated formaldehyde reagent and diluent were additive.

Attainment of equilibrium between cytidine-5′-phosphate and formaldehyde: The spectra of samples of a solution $2.79 \cdot 10^{-3}$ M with respect to cytidine-5′-phosphate, 2.4 M in formaldehyde, buffered to pH 8.3 with 0.1 M phosphate were measured on a Beckman Model DK2 Spectrophotometer after periods of incubation at either room temperature or 37°. Following determination of the spectrum of each sample, its pH was checked on a Beckman Model G pH meter.

Effect of formaldehyde on the dissociation of the amino group of cytidine-5′-phosphate: The pK_a of the cytidylic acid amino group in the presence of formaldehyde (the pG_f) was determined in a manner analogous to that used by LEVY[6] for amino acids, except that reagent-grade aqueous 36–38 % formaldehyde solutions purified in the manner described above were used rather than solutions of the freshly-distilled gas dissolved in water. A series of solutions of cytidine-5′-phosphate and sodium chloride ($4.4 \cdot 10^{-3}$ M and 0.035 M, respectively) varying in formaldehyde concentration from 0 to 10.42 M were prepared by diluting aliquots of a stock solution of cytidine-5′-phosphate at pH 4.45 containing sodium chloride with appropriate volumes of boiled deionized water and 12.72 M formaldehyde. Under these conditions, the pH of a solution diluted only with the water was 4.50. The pH of each solution was measured at room temperature on a Beckman Model G pH meter after 3 h incubation at 37° in a tightly closed container. No additional change in the pH of any of the solutions was observed after 20 h further incubation at 37°.

Potentiometric titrations: Titrations of 1.25-ml samples were conducted under a slow stream of nitrogen gas in a closed (but not airtight) vessel equipped with small magnetic stirrer (Tri-R Instruments, 144-13 Jamaica Avenue, Jamaica 35, N. Y.). Inserted in the vessel were the tip of a capillary microburet of the Kirk-Grunbaum type (Microchemical Specialties Company, 1834 University Avenue, Berkeley 3, Calif.) and the glass and calomel electrodes of a Beckman probe assembly. The electrodes were connected to a Beckman Model G pH meter. After standardization of the meter with pH 7.0 buffer, a measured volume (1.00 ml) of test solution (cytidine-5′-phosphate or cytidine-5′-phosphate plus four molar equivalents of neutralized cytidine sulfate, sodium chloride and formaldehyde, pH 8.5) was introduced into the chamber. At the outset of each experiment, before dilution with formaldehyde, the cytidine-5′-phosphate concentration in each case approximated $3 \cdot 10^{-3}$ M; that of sodium chloride 0.07 to 0.08 M. Prior to its introduction into the chamber each test solution had been made approximately 2 M with respect to formaldehyde by the addition of a measured volume of the concentrated reagent, and had undergone a period of incubation of from 40 to 50 min at room temperature (in one case, 37°) to insure establishment of equilibrium between the substrate and formaldehyde. In order to insure immersion of the electrodes to an adequate depth at the outset of the titration, 0.25 ml of formaldehyde diluted with water to the same concentration as that of the test solution was added, plus sufficient carbonate-free standard sodium hydroxide (10–25 µl) to bring the pH of the solution up to 9. The solution was then titrated with standard hydrochloric acid, the pH being recorded after the delivery of each small volume from the microburet. Stirring was momentarily discontinued at those times when the pH was measured, but resumed during and for a short time after the introduction of the next increment of acid. After the completion of any titration, the standardization of the meter was rechecked with pH 7.0 buffer. Results of the titration were disregarded if the pH of the buffer failed to read 7.00 ± 0.02.

In each experiment a blank, consisting of a solution of sodium chloride and formaldehyde alone carried through an identical incubation procedure was titrated, and in most cases a control titration was performed on a solution consisting of the nucleotide or nucleotide–nucleoside mixture, salt, a volume of water equal to the volume of formaldehyde solution added to the experimental solution, plus whatever volume of standard sodium hydroxide that had been found necessary to bring the initial pH of the corresponding experimental solution to 9.

The amount of cytidine-5′-phosphate in the solution titrated was calculated on the basis of the concentration of total organic phosphorus in the test solution prior to its dilution with formaldehyde, assuming the volumes to have been additive on dilution. Total organic phosphorus was determined after hydrolysis to inorganic phosphate with perchloric acid according to the procedure of ALLEN[7], followed by a phosphomolybdate determination of inorganic phosphate using ascorbic acid as the reducing agent[8,9].

Cytidine concentrations were calculated from the absorbance at 280 mμ of a sample diluted with pH 2 buffer, assuming an ε_{280} of $13.0 \cdot 10^3$ cm/M (ref. 10).

RESULTS AND DISCUSSION

In Table I are recorded the results of the experiment on the time required for the attainment of equilibrium between cytidine-5′-phosphate and formaldehyde. From the table it is evident that the reaction has reached equilibrium within 40 min at room temperature at pH 8.3. The errors of the experiment are such that it cannot be said with certainty that equilibrium at room temperature is reached within 10 min. It seems probable that the reaction between formaldehyde and cytidine-5′-phosphate is significantly slower than that between formaldehyde and the amino acids, which is reported[11] to reach equilibrium in periods of the order of 30 sec to 2 min. In the titration experiments subsequently carried out the nucleotide derivatives were incubated with formaldehyde for at least 40 min under conditions of temperature and pH which had been demonstrated to convert no significant amount of formaldehyde to formic acid.

TABLE I

ABSORBANCE RATIOS FOR CYTIDINE-5′-PHOSPHATE AFTER REACTION WITH FORMALDEHYDE AT pH 8.3

Formaldehyde concentration	*Time (min)*	*Incubation temperature*	A_{290}/A_{275}	A_{250}/A_{280}
0	—	—	0.905	0.847
0	—	—	0.906	0.860
2.4	10	25°	0.707	0.825
2.4	40	25°	0.688	0.797
2.4	30	37°	0.686	0.803
2.4	90	37°	0.686	0.803
2.4	150	37°	0.689	0.802
2.4	270	37°	0.689	0.802

The results of the experiment on the effect of formaldehyde concentration on the pK_a of the cytidylic acid amino group are plotted in Fig. 1. These results are of some theoretical interest in addition to their practical significance with regard to the question of the extent to which the pK_a is lowered. According to the theoretical treatment of the reaction between formaldehyde and amino acids made by LEVY[8], those amino acids which are primary amines (alanine, valine, etc.) at high formaldehyde concentrations obey the equation:

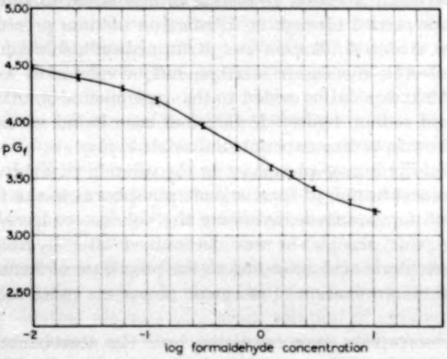

Fig. 1. Effect of formaldehyde on the dissociation of the amino group of cytidine-5′-phosphate. The abscissa represents the logarithm (to the base 10) of the formaldehyde concentration; the ordinate the pK_a (pG_f) of the amino group of cytidine-5′-phosphate at the corresponding formaldehyde concentration. Cytidine-5′-phosphate concentration, $4.4 \cdot 10^{-3}$ *M*; sodium chloride, 0.035 *M*.

$$pK_a - pG_f = \log L_2 + 2 \log [\mathrm{HCHO}]$$

where pK_a has its usual significance, being the negative logarithm (to the base 10) of the dissociation constant of the charged amino group in aqueous solution, while pG_f represents the analogous quantity in a solution containing formaldehyde, [HCHO] represents the molarity of formaldehyde in solution, and L_2 is a constant. Thus a plot of pG_f *versus* the logarithm of the formaldehyde concentration in the case of most of the simple amino acids approaches a straight line with slope of −2 at high formaldehyde concentrations. In the case of the amino acids that are secondary amines (such as proline or sarcosine) however, the theoretical equation obeyed at high formaldehyde concentrations is:

$$pK_a - pG_f = \log L_1 + \log [\mathrm{HCHO}]$$

L_1 being a constant. The curve given by plotting pG_f against log [HCHO] at high formaldehyde concentrations approaches a straight line with slope −1.

Fig. 1, the curve given by the nucleotide, appears at formaldehyde concentrations greater than about 0.1 *M*, closely to approximate a straight line with slope of −0.6. Since there is a suggestion of decrease in the slope at the higher of the formaldehyde concentrations investigated, it is possible that buffering impurities present in the formaldehyde may have served to reduce the limiting slope of the curve somewhat.

It seems unlikely that much of the flattening of the curve is due to buffering by the primary phosphoryl dissociation of the nucleotide, which has a pK_a value of[12] approximately 0.8, except possibly at the very highest of the formaldehyde concentrations employed. The curve seems to indicate that cytidine-5′-phosphate combines with one rather than with two moles of formaldehyde, *i.e.*, it behaves more as though it had a secondary, rather than a primary amino group.

The extent of the depression of the pK_a of the cytidylic acid amino group produced at workable concentrations of formaldehyde was much less than has been observed with the amino acids. Nevertheless it was felt that the shift observed at high formaldehyde concentrations was sufficient to permit the determination of phosphate end-groups by formol titration, at least in some cases.

The titration experiments were performed on solutions which approximated the concentration of monoesterified phosphate that would be present in a decanucleotide at a concentration of 1 mg phosphorus/ml. Titration studies with cytidine-5′-phosphate alone would then be equivalent to titration of a decanucleotide with but a single cytidylic acid residue; when four moles of cytidine/mole of cytidylic acid were also present the solution approximated a decanucleotide containing five cytidylic acid residues per molecule. (This latter solution, of course, also approximates a pentacytidylic acid at half as high a concentration.)

The result of the titration of cytidine-5′-phosphate alone in 2 *M* formaldehyde, plotted as pH *vs* milliequivalents of acid consumed from pH 8.5 to pH 2.25 is given in Fig. 2. (The titration curves of all solutions containing formaldehyde coincide within experimental error above pH 8.5.) In addition to the experimental curve (curve 1), the curve given by the corresponding control solution (substrate but no formaldehyde, curve 2) and those given by the two blank solutions (one with formaldehyde, curve 3, and the other without, curve 4) are shown. Upon comparing the titration curves of the nucleotide with and without formaldehyde (curves 1 and 2) it is obvious that the presence of formaldehyde has effected a detectable separation of the amino and secondary phosphoryl dissociations.

In Fig. 3 are shown the results of the titration of cytidine-5′-phosphate in 2 *M* formaldehyde corrected for the acid consumed in titrating the blank alone (curve 1), and the corresponding curve given when cytidine-5′-phosphate plus four molar equivalents of cytidine are titrated under similar conditions (curve 2). In both experiments the milliequivalents of acid consumed in titrating the substrates from pH 8.5 to 5.0 and from pH 8.5 to 4.5 can be read directly from the curves, and are recorded in Table II. Also recorded in Table II are the milliequivalents of monoesterified phosphate calculated to have been present from the phosphorus analysis of the solutions.

The agreement between the milliequivalents of acid consumed in the titration and the milliequivalents of secondary phosphate present is close in the case where the mononucleotide alone is the substance titrated. The inflection point of the curve appears to be located at pH 4.5 or slightly below, and the milliequivalents of acid consumed in titrating to this pH exceeds the milliequivalents of secondary phosphate calculated to have been present from the phosphorus analysis by only about 5% (close to the limit of accuracy of the method of phosphorus analysis employed). The pH at which the milliequivalents of acid consumed is stoichiometrically equivalent to the secondary phosphate present lies slightly below pH 5.0.

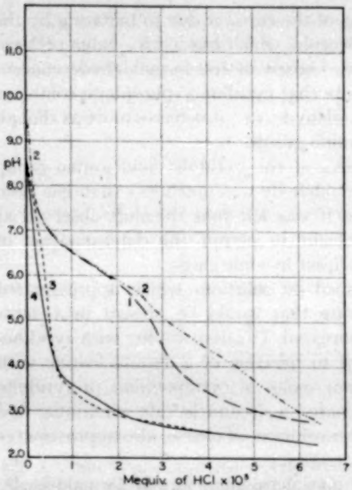

Fig. 2. Titration of cytidine-5′-phosphate in the presence and absence of 2 *M* formaldehyde. Curve 1: $1.84 \cdot 10^{-3}$ *M* cytidine-5′-phosphate, 0.043 *M* in NaCl, 2.30 *M* in HCHO, pH 8.5. Curve 2: $1.84 \cdot 10^{-3}$ *M* cytidine-5′-phosphate, 0.043 *M* in NaCl, no formaldehyde present, alkali equivalent to that necessary to bring the pH of the formaldehyde-containing solution to pH 8.5. Curve 3: (blank for Curve 1): 2.30 *M* formaldehyde, 0.043 *M* in NaCl, pH 8.5. Curve 4: (blank for Curve 2): 0.05 *M* NaCl, same initial pH as curve 2.

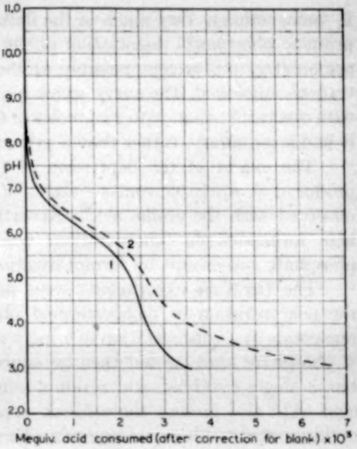

Fig. 3. Titrations conducted in 2 *M* formaldehyde. Curve 1: $1.84 \cdot 10^{-3}$ *M* cytidine-5′-phosphate, 0.043 *M* in NaCl, 2.30 *M* in HCHO. Curve 2: $1.91 \cdot 10^{-3}$ *M* cytidine-5′-phosphate, $7.83 \cdot 10^{-3}$ *M* cytidine 0.044 *M* NaCl, 2.26 *M* HCHO.

TABLE II

DETERMINATION OF NUCLEOSIDE PHOSPHATE BY TITRATION IN 2.3 *M* FORMALDEHYDE

Substances titrated	*Mequiv. nucleoside phosphate titrated* 10³*	*Titration to pH*	*Mequiv. acid consumed × 10³*	*Per cent difference*
Cytidine-5′-phosphate	2.31	5.0	2.27	—1.73
		4.5	2.42	5.19
Cytidine-5′-phosphate plus cytidine	2.21	5.0	2.62	18.6
		4.5	2.95	33.5

* Calculated from phosphorus analysis.

When four molar equivalents of cytidine are present in addition to the cytidylic acid, however, the disagreement between the milliequivalents of acid consumed in the titration and the value calculated from the phosphorus analysis is substantial. The inflection point appears to be located between pH 4.5 and 5.0, yet the milliequivalents of acid consumed in titrating to pH 5.0 exceeds the milliequivalents of nucleotide by nearly 19 %.

In the aforementioned experiment on the effect of formaldehyde on the pK_a of the amino group of cytidine-5′-phosphate it had been found that the pK_a value was lowered to 3.46 or below at the formaldehyde concentration employed in the titration experiment (2.27 *M*). It can be calculated from the HENDERSON-HASSELBACH equation that the milliequivalents of acid consumed in titrating to pH 5.0 a solution of the salt of an acid with pK_a 3.50 (at the same concentration as that of the amino groups in the quoted titration experiment) are $3.47 \cdot 10^{-4}$. This amount represents a considerable fraction (nearly 16%) of the total milliequivalents of nucleoside phosphate present ($2.21 \cdot 10^{-3}$), but should represent an upper limit of error from this source, since the true pK of the amino group is believed to have been below 3.50. The actual error observed is, of course, of this order of magnitude, but is significantly higher than this calculated upper limit.

It will be noted that the milliequivalents of acid consumed in titrating the nucleotide–nucleoside mixture (curve 2, Fig. 3) from pH 8.5 to 7.0 exceeds the milliequivalents consumed in titrating the nucleotide alone (curve 1) to the same pH by about $0.09 \cdot 10^{-4}$. This difference is probably close to within the margin of experimental error, but if any portion of it is due to contamination of the mixture solution with a buffer (such as bicarbonate), it would account in part for the discrepancy.

Another possible explanation for the magnitude of the observed discrepancy is that the pK_a of the amino group of the free nucleoside, cytidine, may not be lowered to the same extent at a given formaldehyde concentration as that of the amino group of cytidine-5′-phosphate, despite the fact that the pK_a of the amino group of the nucleoside in aqueous solution is lower than that of the nucleotide[10, 13] (4.22 *vs* 4.5). Whether or not such is the case could be determined by a direct experiment with cytidine and formaldehyde, but the question is irrevelant to the problem of polynucleotide end-group determination, since cytidine itself is not a component of polynucleotides.

The results obtained when cytidine-5′-phosphate-cytidine mixtures were titrated in formaldehyde make it appear doubtful that the titration method with formaldehyde is applicable to many polynucleotides, since most large polynucleotides of natural origin would contain several times as many amino groups as terminal phosphate groups. However, the possibility remains that the behavior of the amino group of the free nucleoside toward formaldehyde is sufficiently different from that of a cytidylic acid residue in a polynucleotide chain for such a method to be useful to a limited extent. Interference due to the dissociation of amino groups should be further reducible by the use of formaldehyde concentrations higher than were used in the titration experiments reported here, although practical considerations place limits on the formaldehyde concentrations that can be used.

An ideal test substrate for the method would be an oligonucleotide of, say, ten residues in length, five of which were cytidylic (or deoxycytidylic) acid residues. It is probable that synthetic methods for such substances will be developed in the relatively near future[14].

ACKNOWLEDGEMENTS

The advice and encouragement rendered him on many occasions by Dr. R. L. SINSHEIMER is gratefully acknowledged. The author is a Public Health Service Research Fellow of the National Cancer Institute.

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ÉTUDES CINÉTIQUES SUR LA GALACTOSIDE-PERMÉASE D'*ESCHERICHIA COLI*

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Reçu le 12 Août, 1959)

SUMMARY

Kinetic studies on the galactoside-permease in E. coli

Galactoside-permease positive *E. coli* accumulates radioactive thiogalactosides in the intracellular space until a steady state is reached. Measurement of the steady state concentration level, of the affinity, and of the rate of turnover in the steady state makes possible the calculation of the activity of the mechanism of uptake and of the rate constant of passive exit. The entry mechanism is catalytic; it is coupled with metabolic energy donors. The energy consumption corresponds to the hydrolysis of one mole of ATP per mole of TMG flowing inward. Passive exit is not by free diffusion, but can be understood as a carrier diffusion.

A model is proposed which is compatible with the observed behaviour of the entry and exit parameters under various conditions.

INTRODUCTION

Un nombre sans cesse croissant de phénomènes de perméation sélective, de transport actif à travers des membranes biologiques pluricellulaires, de concentration ou

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Abréviations utilisées: TMG, méthyl-thio-β-D-galactoside; TDG, galactosyl-thio-β-D-galactoside; TPG, phényl-thio-β-D-galactoside; DNP, 2,4-dinitrophénol; *p*CMB, *p*-chloro-mercuribenzoate; ATP, acide adénosine triphosphorique.

d'extrusion de substances par des cellules ont été décrits. L'importance de ces phénomènes dans les régulations biologiques est évidente. Le mécanisme de tels phénomènes a fait l'objet de nombreuses spéculations, cependant les structures essentielles pour la sélectivité de la perméation ou pour le couplage énergétique, dans le cas de transport actif, n'ont jamais pu être identifiées.

Les perméases bactériennes décrites par RICKENBERG, COHEN, MONOD ET BUTTIN[1] offrent un sujet particulièrement favorable à l'étude d'un tel mécanisme. L'objet du présent travail, la galactoside-perméase, de par sa stéréospécificité, par sa synthèse inductible et par l'existance de mutants simples est particulièrement bien individualisée. Elle offre en outre des facilités techniques grâce à l'utilisation de thiogalactosides non métabolisables et radioactifs, et grâce au débit élevé de perméation qui permet de mesurer les besoins énergétiques du processus.

On sait[1] que la concentration de substrat intracellulaire en régime stationnaire obéit à la relation*:

$$G_{\text{in}} = Y \frac{G_{\text{ex}}}{K_m + G_{\text{ex}}} \tag{1}$$

qui pouvait résulter soit de l'adsorption des galactosides sur Y sites spécifiques (μmoles/g) avec une constante d'affinité de $1/K_m$ soit d'un mécanisme d'entrée catalytique obéissant à la loi de Michaelis avec une constante K_m. La vitesse de cette réaction d'entrée

$$V_{\text{in}} = V_{\text{in}}^{\text{max}} \frac{G_{\text{ex}}}{K_m + G_{\text{ex}}} \tag{2}$$

doit être compensée en régime stationnaire par une sortie passive proportionnelle au gradient de concentration**

$$V_{\text{ex}} = G_{\text{in}}\, k_{\text{ex}} \tag{3}$$

En régime stationnaire $V_{\text{in}} = V_{\text{ex}}$ donc

$$G_{\text{in}} = \frac{V_{\text{in}}^{\text{max}}}{k_{\text{ex}}} \, \frac{G_{\text{ex}}}{K_m + G_{\text{ex}}} \tag{4}$$

Cette équation est formellement identique à (1) sauf que Y, la capacité à saturation n'est plus un paramètre intrinsèque du système mais un quotient $V_{\text{in}}^{\text{max}}/k_{\text{ex}}$ pouvant être variable.

Les premières études cinétiques ont permis d'éliminer l'hypothèse d'adsorption et d'étayer l'hypothèse d'un mécanisme catalytique[2].

L'étude des vitesses d'entrée, de renouvellement et de sortie dans des conditions variées peut alors mettre en évidence des variations indépendantes de ces processus, l'entrée catalysée et la sortie passive, et jeter quelque lumière sur leur nature.

La constatation d'une consommation accrue d'oxygène liée au fonctionnement de la perméase[3] entraîne la question: la concentration des galactosides par la perméase est-elle un transport actif? ou en termes plus concrets; les galactosides accumulés sont-ils sous la même forme moléculaire que dans le milieu, l'énergie dépensée apparaît-elle sous forme de travail osmotique ou sous forme d'énergie de liaison avec un constituant cellulaire à définir? Rien ne permet à l'heure actuelle d'affirmer la seconde hypothèse. Par contre la contribution osmotique des galactosides intracellulaires[4] est un fort argument en faveur du transport actif.

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* Symboles utilisés: G, un β-galactoside ou β-thiogalactoside, substrat de la perméase ou sa concentration. G_{in}, G_{ex}, concentration dans le milieu intracellulaire ou extracellulaire. K_m, concentration de demi-saturation. Y, $G_{\text{in}}^{\text{max}}$, concentration intracellulaire à l'équilibre pour une concentration externe saturante ou "capacité spécifique". V, vitesse de réaction; k, constante de vitesse de réaction. Les indices "in" et "ex" se réfèrent à des réactions d'entrée ou de sortie respectivement. $t_{1/2}$, temps de demi-équilibration ou demi-échange.

** On néglige G_{ex} devant G_{in}. Ceci introduit une erreur de l'ordre de 1 % ou moins.

Enfin la pénétration passive des galactosides dans les cellules dépourvues de perméase pouvait offrir un modèle de perméation passive dont la comparaison avec le mécanisme passif de sortie des galactosides dans les cellules où la perméase fonctionne pouvait être fructueux.

MATÉRIEL ET MÉTHODES

Les expériences rapportées ont été faites sauf indication contraire avec la souche ML308 à perméase constitutive et la souche ML3 dépourvue de perméase.

Les microbes sont cultivés en milieu minéral liquide avec du succinate de potassium ou du glucose comme source de carbonne et agités à 34°. Ils sont récoltés par centrifugation au cours de la phase de croissance exponentielle et resuspendus à une densité de 200 μg/ml environ dans du milieu minéral, additionné de succinate de potassium à 4 g/l et de chloromycétine à 50 μg/ml. La suspension est agitée à la température de l'expérience au moins 15 min avant l'addition de substrat.

Les substrats utilisés ont été: Le méthylthio β-galactoside ou TMG; le β-D-thiogalactoside β-D-galactoside (thiodigalactoside) TDG; le phénylthio β-D-galactoside TPG; le lactose (glucose 6-β-D-galactoside).

Les thiogalactosides radioactifs ont été marqués par ^{35}S (TDG et TPG) ou par ^{14}C dans le méthyle (TMG).

A des temps variables après l'addition du substrat radioactif à la suspension agitée des portions de 1 ml sont prélevées et filtrées sur filtres millipore. Préalablement le filtre mis en place est recouvert de 5–6 ml de milieu minéral à 0°. La suspension mélangée à ce volume est instantanément refroidie. Ce moment considéré comme le temps exact du prélèvement est défini à 2–3 sec près. La filtration est suivie de deux rinçages avec 2 ml de milieu glacé. Le milipore est ensuite séché et sa radioactivité est mesurée sans autre manipulation. La concentration intracellulaire de lactose a été mesurée, après élution à l'eau bouillante, par la méthode de SOMOGYI-NELSON.

La consommation d'oxygène a été mesurée par l'électrode à goutte de mercure à tension constante — 0.6 V contre l'électrode au calomel saturé.

Le dégagement de ^{14}CO$_2$ a été mesuré après entraînement par air décarbonaté sur des microabsorbeurs de soude.

RÉSULTATS

Renouvellement

La Fig. 1 représente une expérience cinétique d'accumulation (A) et d'échange (B et C) en régime stationnaire du TMG à la concentration de $1 \cdot 10^{-3}$ *M*. Pour ces deux dernières (B et C) le TMG non radioactif est ajouté aux microbes au temps 0, et le TMG marqué après 30 et 160 min respectivement, sans changer la concentration finale de TMG. On observe: (a) Que la totalité du TMG est échangeable, les plateaux des courbes A, B et C se rejoignent. (b) Que l'échange se fait à la même vitesse que l'équilibration initiale. La vitesse de la réaction d'entrée est donc pratiquement indépendante de la concentration intracellulaire.

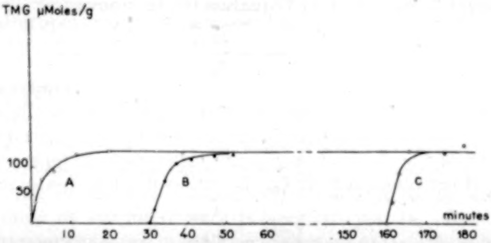

Fig. 1. Cinétique d'entrée du TMG (A) et cinétique d'échange en régime stationnaire (B et C) ML 308 suspendu dans un milieu minéral maltosé. Concentration de TMG 10^{-3} *M* partout température, 15°.

Détermination des paramètres

La Fig. 2 représente une expérience d'échange de TDG en régime stationnaire à deux concentrations différentes:

On voit que les pentes initiales sont proportionnelles aux concentrations atteintes à l'équilibre.

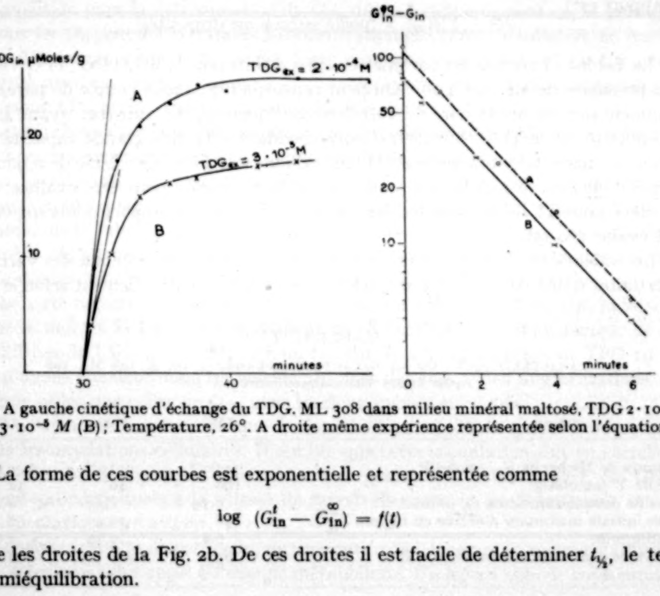

Fig. 2. A gauche cinétique d'échange du TDG. ML 308 dans milieu minéral maltosé, TDG $2 \cdot 10^{-4}$ *M* (A) et $3 \cdot 10^{-5}$ *M* (B); Température, 26°. A droite même expérience représentée selon l'équation (5).

La forme de ces courbes est exponentielle et représentée comme

$$\log \ (G_{\text{in}}^{t} - G_{\text{in}}^{\infty}) = f(t)$$

donne les droites de la Fig. 2b. De ces droites il est facile de déterminer $t_{1/2}$, le temps de demiéquilibration.

La vitesse initiale d'échange ou la pente à l'origine des courbes de la Fig. 2a

$$V_{\mathrm{in}} = G_{\mathrm{in}}^{\infty} \frac{\ln 2}{t_{1/2}} \tag{5}$$

En comparant l'équation (4) et l'équation (5), on trouve

$$k_{\mathrm{ex}} = \frac{\ln 2}{t_{1/2}} \tag{6}$$

On voit donc que si la mesure des concentrations intracellulaires en régime stationnaire en fonction de la concentration extracellulaire de galactoside permet la détermination graphique de Y et de K_m une expérience cinétique permet la détermination de $t_{1/2}$ et par conséquent de k_{ex}. La vitesse d'entrée à saturation est donnée par:

$$V_{\mathrm{in}}^{\max} = Y\, k_{\mathrm{ex}} \tag{7}$$

G_{ex} et K_m sont exprimés en moles/l, G_{in} et Y en µmoles/g de matière sèche microbienne.

Si on veut considérer le rapport des concentrations extra et intra cellulaires, il faut faire intervenir le volume disponible.

La limite supérieure du rapport de concentration est atteinte pour les faibles valeurs de G_{ex} et G_{in}, elle est égale à la tengante à l'origine de la courbe hyperbolique.

$$G_{\mathrm{in}} = f\,(G_{\mathrm{ex}}),\ \text{equation (1)}$$

ce rapport est:

$$R = (Y|K_m) \times (\text{poids sec}|\text{volume disponible}) \tag{8}$$

Le Tableau I résume les paramètres ainsi définis pour TMG, TDG, TPG vis-à-vis de la perméase de ML 308 à 26°. On peut remarquer qu'aucun couple de paramètres fondamentaux ne présentent de corrélations évidentes. Le substrat ayant la plus forte affinité est le TDG, le substrat correspondant à la plus grande capacité est le TMG et le substrat dont la sortie est la plus rapide est le TPG. La vitesse de renouvellement du TPG est si rapide que seule une limite inférieure peut être évaluée. R est plus élevé pour TDG que pour les deux autres substrats (le rapport poids sec/volume a été évalué à 0.25).

Les valeurs de Y et les paramètres qui en découlent sont sujets à des variations importantes d'une culture à l'autre, selon les souches, et naturellement selon le degré

TABLEAU I

CARACTÉRISTIQUES DE LA GALACTOSIDE-PERMÉASE DE *E. coli* ML 308
POUR TROIS THIOGALACTOSIDES À 26°

Substrat	*TMG*	*TDG*	*TPG*
Constante de Michaelis K_m (moles/l)	$5 \cdot 10^{-4}$	$2 \cdot 10^{-5}$	$2.5 \cdot 10^{-4}$
Capacité Y (µmoles/g)	160	40	32
Temps de demiéquilibration $t_{1/2}$ (min)	0.75	1.35	< 0.25
Vitesse initiale maximum d'entrée en régime $V_{\mathrm{in}}^{\max}$	148	20.4	> 86
k_{ex} (ml/g/min)	0.82	0.59	> 2.7
Rapport de concentration maximum R	65	400	26

d'induction pour les souches inductibles, Les valeurs relatives de ces paramètres pour les trois substrats, les K_m et les $t_{1/2}$ par contre sont des caractéristiques du système. L'accumulation du lactose par la souche W2244 qui ne l'utilise pas, atteint 550 μmoles/g à $+ 4°$ pour une concentration de $5 \cdot 10^{-3}$ M dans le milieu, soit 20 % de la matière sèche cellulaire.

La chromatographie sur papier a montré que le seul sucre réducteur détectable était le lactose.

Les besoins énergétiques

L'accumulation des galactosides étant inhibée par les inhibiteurs de la phosphorylation oxydative comme le 2,4-DNP ou l'azide de sodium, l'influence du fonctionnement de la galactoside-perméase sur la consommation d'oxygène a été mesuré par l'électrode à goutte de mercure. La chloromycétine a été omise au cours de ces expériences à cause de son interférence polarographique. De même a été omis le substrat carboné pour éviter que le faible accroissement de Q_{O_2} dû au fonctionnement de la perméase ne soit masqué par une respiration de base intense. Les fluctuations de ces mesures sont beaucoup plus importantes que pour la détermination de l'activité de la perméase.

Dès l'addition du thiogalactoside, la respiration s'accélère et reste constante ensuite pendant 30 à 40 min.

Si la concentration de thiogalactoside n'est pas saturante pour la perméase, une addition supplémentaire provoque une nouvelle augmentation du Q_{O_2}.

Le Q_{O_2} endogène, 5 à 10 μmoles/g de matière sèche par minute, est soustraite par convention pour la détermination du Q_{O_2} dû au fonctionnement de la perméase.

Tous les Q_{O_2} ont été extrapolés à concentration saturante de substrat, en prenant les K_m déterminés pour la perméase. Leur moyenne pour TMG est de 8.9 μmoles d'oxygène avec une erreur standard $\pm$ 3 μmoles/g/min pour 16 déterminations. La valeur pour TPG est de 15.0 $\pm$ 3.4 μmoles/g/min sur 12 déterminations.

Les souches dépourvues de perméase, ML 30 non induit ou ML 3, ne présentent aucun accroissement de leur Q_{O_2} par addition de thiogalactoside.

Le parallélisme de l'"extra-oxygène" avec le fonctionnement de la perméase est donc à la fois qualitatif et quantitatif. Pour exclure la possibilité qu'une impureté présente dans le substrat soit la cause de cette consommation d'oxygène, nous avons examiné le dégagement de CO_2 pour savoir s'il provenait du substrat ou des microbes.

Ce dégagement de $^{14}CO_2$ d'une suspension lavée de microbes, cultivés sur [^{14}C]-fructose a été mesuré en l'absence et en présence de TMG et de TPG non radioactifs. Comme le montre la Fig. 3 le dégagement de $^{14}CO_2$ s'est accru d'un facteur de 1.75 par addition de TMG $2 \cdot 10^{-3}$ M, et d'un facteur de 2.5 par addition de TPG 10^{-3} M (nous n'avons pas déterminé les valeurs absolues des Q_{CO_2}). Ces augmentations sont du même ordre que celles du Q_{O_2} pour les deux mêmes substrats.

Ces expériences montrent que le fonctionnement de la galactoside-perméase stimule les oxydations cellulaires. Il semble que cette stimulation soit en corrélation avec les besoins énergétiques du transport de galactoside. D'autre part, ces besoins semblent proportionnels à la vitesse de renouvellement, ils ne cessent pas, une fois le régime stationnaire atteint.

Cependant il n'était pas exclu, qu'une partie de l'échange en régime stationnaire put se faire sans faire appel à l'énergie métabolique. Un léger excès de consommation d'oxygène, limité à la première minute des mesures, a pu échapper à l'observation.

Une expérience cinétique avec 2,4-dinitrophénol comme inhibiteur permet de trancher ce problème. La Fig. 4 représente une telle expérience. On voit que 5 min après l'addition du DNP l'échange représente encore 62 % de celui observé avant l'addition de l'inhibiteur, alors qu'après le même temps d'action du DNP la vitesse d'entrée est réduite à 28 % du témoin. Si on considère que la source d'énergie résiduelle est la même dans les deux expériences, c'est-à-dire suffisante pour assurer 28 % de la vitesse initiale on arrive à la conclusion qu'une fraction de l'ordre de 34 % de l'échange en régime stationnaire peut se faire sans nouvel apport d'énergie métabolique. Si on transpose ce résultat au TMG dans les conditions des mesures respirométriques, on trouve un rapport TMG/O = 60/(17.8 ± 6) = 3.8 ± 1.3. Si on considère que V_{in} du TMG est plutôt surévalué dans les conditions des expériences respirométriques en carence carbonée, on doit assigner au rapport TMG/O la valeur la plus probable de 3 qui pourrait refléter l'utilisation des liaisons pyrophosphate de haute énergie dans le processus de transport.

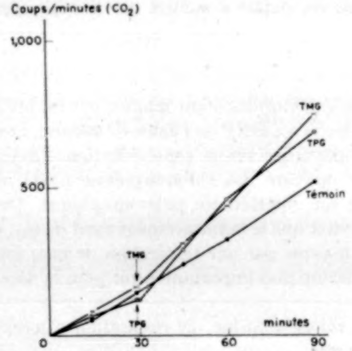

Fig. 3. Dégagement de $^{14}CO_2$ en fonction du temps. ML 308 cultivée sur [^{14}C]fructose lavé et suspendu dans un milieu minéral sans source de carbone extérieure. Température, 21°. TMG $2 \cdot 10^{-3}$ *M*, TPG 10^{-3} *M*.

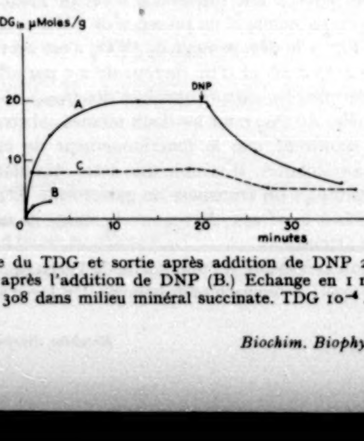

Fig. 4. Cinétique d'entrée du TDG et sortie après addition de DNP $2 \cdot 10^{-3}$ *M* (A). Cinétique d'entrée du TDG 5 min après l'addition de DNP (B.) Echange en 1 min dans des conditions identiques à A (C) ML 308 dans milieu minéral succinate. TDG 10^{-4} *M*; Température, 26°.

Coefficient de température

La Fig. 5 représente deux expériences de renouvellement, de TMG d'une part, de TDG d'autre part à trois températures 34°, 24° et 14°. Il est frappant de voir que si la vitesse d'entrée des deux substrats varie, avec un Q_{10} de l'ordre de 2, la concentration atteinte en régime stationnaire est pratiquement indépendante de la température pour TDG, tandis qu'elle varie pour TMG en sens inverse de la température.

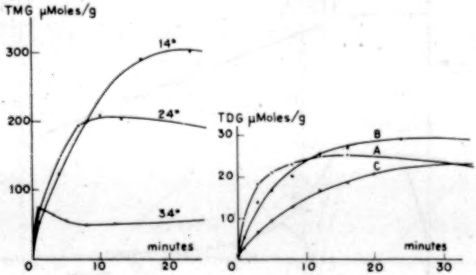

Fig. 5. Cinétique d'échange du TMG (10^{-3} *M*) et du TDG (10^{-4} *M*) à 3 températures différentes. ML 308 dans milieu minéral succinate.

On peut en déduire que la constante de vitesse de sortie a pour TDG un Q_{10} à peu près égal à celui de la vitesse d'entrée, alors que la sortie de TMG a un Q_{10} de l'ordre de 4.

Inhibiteurs

On a vu que sous l'action du 2,4-DNP, et il en est de même pour l'azide de sodium, l'accumulation des thiogalactosides est inhibée. Si l'inhibiteur est additionné après l'accumulation, la concentration intracellulaire baisse lentement. Ces agents agissent apparemment par inhibition du couplage énergétique et non pas directement sur le catalyseur spécifique. Les réserves cellulaires en composés pyrophosphoriques riches en énergie ainsi que la possibilité d'échanges avec conservation d'énergie peuvent expliquer la lenteur de l'effet observé. Un inhibiteur direct du mécanisme catalysant l'entrée devrait provoquer une sortie exponentielle ayant la même constante de temps que l'échange en régime stationnaire.

Le *p*-chloromercuribenzoate (*p*CMB) est un inhibiteur des groupements SH et à ce titre il inhibe de nombreux enzymes métaboliques. Cependant sous son action la vitesse de renouvellement des galactosides par la perméase décroît immédiatement et exponentiellement.

L'inactivation par le *p*CMB est irréversible et totale après un temps d'action suffisante. La vitesse d'inactivation dépend de la concentration de *p*CMB, mais pour une même concentration de l'inhibiteur, elle varie selon qu'un thiogalactoside est présent ou absent. La présence d'un thiogalactoside exerce un effet protecteur à peu près compétitif. Cette protection par le substrat spécifique qui ne réagit pas directement avec le *p*CMB suggère que l'action de ce dernier s'exerce directement sur la molécule porteuse des sites spécifiques, c'est-à-dire, la perméase proprement dite.

La Fig. 6 représente une expérience d'inhibition de la perméase par le *p*CMB à la concentration de 10^{-4} M, Le substrat est le TDG à la concentration de 10^{-4} M. On voit la décroissance exponentielle de la vitesse de renouvellement qui contraste avec la lenteur et l'allure quasi linéaire de la sortie du TDG. Au moment où la vitesse de renouvellement est tombée à une valeur négligeable, la sortie de TDG est environ cinq fois plus lente que l'échange en régime stationnaire.

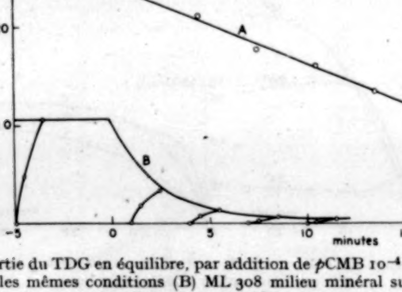

Fig. 6. Cinétique de sortie du TDG en équilibre, par addition de *p*CMB 10^{-4} M (A) Echange après 45 sec et 90 sec dans les mêmes conditions (B) ML 308 milieu minéral succinate TDG 10^{-4} M; Température, 26°.

Compétitions

On a vu au premier paragraphe que le niveau stationnaire est proportionnel à la vitesse de renouvellement pour un substrat donné à une température déterminée, et l'équation (4) montre que le coefficient de proportionnalité est $1/k_{ex}$.

La Fig. 7 représente, selon la méthode de LINEWEAVER ET BURK l'inhibition de la vitesse d'entrée et du niveau stationnaire de TMG par TDG à concentrations variables. La Fig. 8 représente dans les mêmes conditions l'inhibition de la vitesse d'entrée et du niveau stationnaire du TDG par TMG.

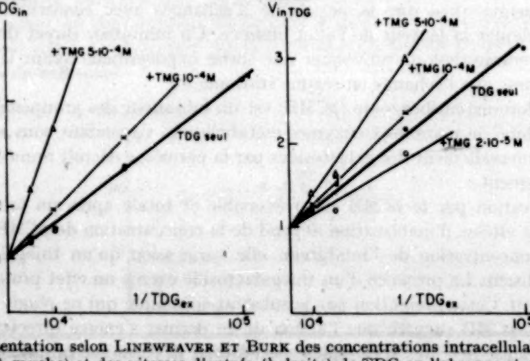

Fig. 7. Représentation selon LINEWEAVER ET BURK des concentrations intracellulaires en régime stationnaire (à gauche) et des vitesses d'entrée (à droite) de TDG en l'absence et en présence de TMG; Température, 26°.

On voit que le TMG inhibe davantage le niveau stationnaire que la vitesse d'entrée du TDG et inversement TDG inhibe davantage la vitesse d'entrée du TMG que son niveau d'équilibre.

Ceci implique que le coefficient de proportionnalité entre niveau d'équilibre et vitesse d'entrée: $1/k_{\mathrm{ex}}$ de chaque substrat est modifié par la présence de l'autre substrat en compétition. L'accélération du renouvellement du TDG en présence de TMG mesurée directement confirme cette interférence.

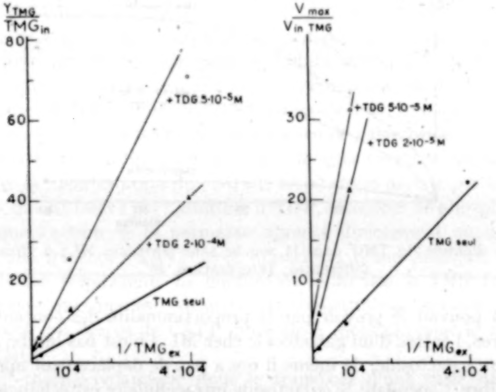

Fig. 8. Représentation selon LINEWEAVER ET BURK des concentrations intracellulaires en régime stationnaire (à gauche) et des vitesses d'entrée de TMG en l'absence et en présence de TDG; Température, 26°.

Une autre anomalie apparaît à l'examen des courbes de la Fig. 7. C'est aux très faibles concentrations de TMG que la vitesse de renouvellement du TDG n'est pas inhibée, mais au contraire, stimulée. Nous essayerons dans la discussion de donner une interprétation de ce phénomène.

Une autre inhibition atypique, celle exercée par le glucose sur la galactoside-perméase a été observée par HORECKER[8]. L'addition du glucose déprime le niveau d'accumulation des thiogalactosides. Une étude cinétique a montré que le glucose intervient essentiellement pour accélérer la sortie du thiogalactoside, son effet sur la vitesse d'entrée est faible ou nul.

Perméation passive dans les souches dépourvues de perméase

ML 3 dépourvue de galactoside-perméase ne concentre pas les galactosides, et la méthode expérimentale a dû être légèrement modifiée: des suspensions environ 10 fois plus concentrées ont été employées avec des prises d'essai de 0.1 ml de sorte que la contamination est diminuée d'un facteur de 10. Elle peut être estimée à moins de 10 % du plateau final.

La Fig. 9 montre la cinétique d'entrée du TMG à 3 concentrations différentes. Trois faits peuvent être déduits de cette expérience. Premièrement, le niveau final de concentration intracellulaire est proportionnel à la concentration dans le milieu.

Ensuite le temps d'équilibration est de l'ordre de 10 fois plus long que celui observé avec la perméase. Enfin, la concentration finale à l'équilibre correspond à un volume disponible de l'ordre de 1 µl/mg de cellules sèches, nettement plus faible que l'espace aqueux habituellement admis: environ 4 µl.

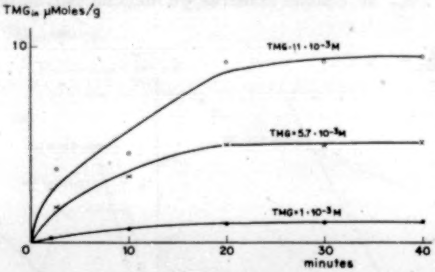

Fig. 9. Cinétique d'entrée du TMG dans la souche sans perméase ML3 à trois concentrations différentes; Température, 26°.

Comme on pouvait le prévoir par la proportionnalité des concentrations extra et intracellulaires, l'entrée d'un galactoside chez ML 3 n'est pas inhibé par la compétition d'un autre galactoside, de même il n'y a pas de déplacement après l'établissement de l'équilibre. Cependant le galactoside intracellulaire est échangeable contre le galactoside dans le milieu sensiblement à la même vitesse que l'entrée initiale.

Les inhibiteurs du métabolisme comme le DNP ainsi que le *p*CMB sont sans effet sur la perméation du TMG comme le montre la Fig. 10. Cette même figure montre que l'addition du glucose diminue rapidement et considérablement le niveau intracellulaire de galactoside. Il s'agit là d'une véritable expulsion active qui est parallèle à l'effet du glucose sur les galactosides activement accumulés dans les souches à galactoside-perméase.

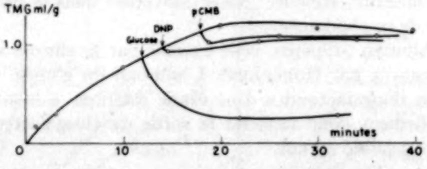

Fig. 10. Cinétique d'entrée du TMG dans la souche sans perméase ML3 (A). Déplacement par addition de glucose $2 \cdot 10^{-2}$ *M* (B). Effet dc DNP $2 \cdot 10^{-3}$ *M* (C) et du pCMB 10^{-4} *M* (D).

DISCUSSION

Il se dégage de l'ensemble des résultats que l'accumulation spécifique de galactoside ne peut être due à l'adsorption sur des sites intracellulaires spécifiques. Il suffit de considérer le Tableau I, qui montre combien est variable la capacité d'accumulation à saturation pour différents substrats, ou encore la Fig. 5 montrant que pour un même substrat la capacité varie largement avec la température. On peut donc affirmer que l'action de la perméase est catalytique et, de plus, que les sites catalytiques spécifiques, gouvernant le phénomène, sont essentiellement accessibles au substrat extracellulaire et largement indépendants de la concentration intracellulaire. C'est pour un site accessible de l'extérieur qu'entrent en compétition les différents galactosides, puisque c'est leur concentration extracellulaire qui régit la compétition. Cette accessibilité de l'extérieur impose à ces sites catalytiques un rôle dans le transport du galactoside du dehors en dedans.

La nature possible d'un catalyseur de transport est jusqu'à nouvel ordre du domaine de la pure spéculation. On peut cependant inférer dans le cas de la galactoside-perméase, que la catalyse est du type enzymatique, étant donné le couplage énergétique. En effet l'utilisation de l'énergie métabolique implique la rupture d'une liaison riche en énergie qui n'a lieu qu'en présence de galactoside.

L'accélération respiratoire provoquée par le galactoside en présence de la perméase peut être la manifestation du contrôle respiratoire décrite par LARDY[7] chez les mitochondries, qui est relevé par l'addition d'ADP, accepteur de phosphate. Il suffirait de supposer que l'action de la perméase aboutit directement ou indirectement à l'hydrolyse d'une molécule d'ATP avec apparition d'une molécule d'ADP ce qui lèverait l'inhibition respiratoire. Le nombre de molécules de TMG transportées par atome d'oxygène consommé est parfaitement compatible avec l'utilisation de l'ATP ou d'un composé qui dérive de l'ATP molécule à molécule pour le transport d'une molécule de TMG.

Le travail thermodynamique pour le transport d'une mole de TDG dont le rapport de concentration est de l'ordre de 400, est de Δ F = 3600 cal/mole donc l'énergie de l'hydrolyse de l'ATP est largement suffisante.

Des unités énergétiques plus petites que l'ATP seraient plus économiques mais si une molécule d'ATP devait donner plus d'une de ces molécules donatrices d'énergie, le rapport TMG/O devrait être au moins égal à 6 ce qui est incompatible avec nos résultats.

L'hypothèse de transport actif est pratiquement inévitable en présence des données actuelles du problème. La contribution osmotique des galactosides intracellulaires ne peut s'expliquer en dehors de cette hypothèse qu'en supposant les galactosides intracellulaires chimiquement modifiés. Or la seule forme modifiée, le dérivé acetylé des thiogalactosides, qui s'observe dans les conditions où la perméase est fonctionnelle, représente une faible fraction des galactosides accumulés. Cette fraction varie considérablement d'un substrat à l'autre. Ce dérivé n'est pas détectable quand la perméase fonctionne en l'absence de source carbone. En tout état de cause les molécules de galactoside ayant subi un ou plusieurs cycles d'entrée et de sortie des cellules sont chimiquement inchangées, alors que le dérivé acétylé ne redonne jamais une quantité détectable du galactoside initial dans ces conditions.

Le modèle qui sans complications inutiles semble rendre compte de toutes nos observations est schematisé Fig. 11. Il représente la perméase comme un enzyme P qui agissant sur une molécule donatrice d'énergie $R \sim T$ et une molécule de galactoside G_{ex} activerait cette dernière. Cette activation pourrait être matérialisée par la combinaison du galactoside avec T, une molécule ou radical transporteur; GT ayant des caractéristiques physicochimiques lui permettant le passage de la barrière qui empêche la traversée du galactoside natif. La réaction $R \sim T + G \longrightarrow GT + R$ serait pratiquement irréversible, et c'est sa vitesse qui est l'étape limitante pour l'ensemble du phénomène. Cette vitesse elle-même dépend essentiellement de la concentration de G_{ex}, et non de celle de $R \sim T$ supposé toujours en excès. Il suffirait que cette molécule GT à l'intérieur de la cellule se désactive spontanément, ou se dissocie de son transporteur (il n'est pas nécessaire de postuler un second enzyme).

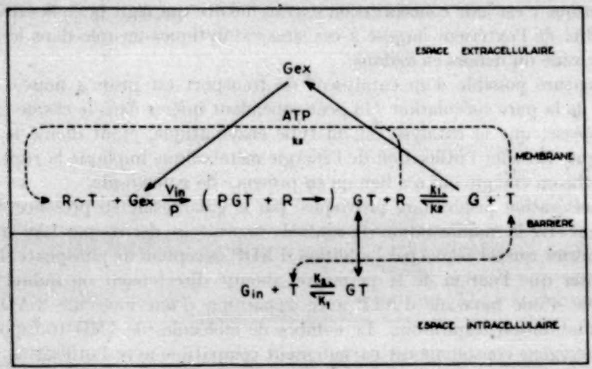

Fig. 11. Modèle de mécanisme proposé.

L'existence éphémère et en quantités catalytiques de la molécule activée expliquerait qu'on n'ait pu la mettre en évidence.

Il y a cependant deux faits qui tendent à montrer l'existence d'un complexe activé du substrat. Le premier est la possibilité d'échange avec conservation d'énergie, plus exactement la différence des degrés d'inhibition par une même dose de DNP de l'entrée et du renouvellement du TDG (Fig. 4). Cet échange peut avoir lieu au niveau du complexe activé lié ou non à l'enzyme. Le second argument peut être tiré du phénomène paradoxal de l'accélération de l'entrée du TDG en présence d'une très faible concentration de TMG (Fig. 7). Comme on sait que l'activation du TMG se fait 8 fois plus rapidement que celle du TDG (Tableau I) il suffit que le TDG en large excès puisse déplacer le TMG activé de son complexe pour pouvoir bénéficier en partie de sa vitesse d'activation, et surcompenser la compétition au niveau de la perméase.

Le mécanisme de sortie passive sous l'influence du gradient de concentration ne poserait pas de problème spécial en soi. Cependant la différence des constantes k_{ex} des souches avec ou sans perméase, l'augmentation de k_{ex} en présence de glucose, son abaissement par les inhibiteurs de l'entrée, le changement de k_{ex} du TDG quand il est en compétition avec TMG semblent exclure une sortie par simple diffusion. On ne voit en effet pas de raison plausible pour que la barrière de perméabilité modifie ses caractéristiques phsyiques de telle sorte que la diffusion du TDG soit accélérée, sans que soit changée celle du TMG.

Notre hypothèse est que la sortie des galactosides s'effectue par un processus de "diffusion facilitée"; c'est-à-dire à l'état complexe avec un transporteur qui rend franchissable la barrière de diffusion. Ce complexe serait dissociable et libérerait le galactoside natif à l'extérieur de la barrière. Dans la Fig. 13, ce complexe est représenté comme identique à GT qui permet l'entrée. Pour cela on suppose que la réaction $GT \rightleftharpoons G + T$ est réversible et spontanée et qu'elle peut avoir lieu sur la face externe de la barrière, aussi bien que sur la face interne. L'équilibre serait de loin en faveur de la dissociation. Cette réaction réversible représente un mécanisme de sortie passive, sous la dépendance du gradient de concentration.

L'état stationnaire est caractérisé par le fait que tous les intermédiaires sont à concentration stable.

Sur la face externe:

$$\frac{d(GT)}{dt} = V_{\mathrm{in}} + k_2 G_{\mathrm{ex}} T - k_1 GT = 0 \qquad (9)$$

Dans l'espace intracellulaire:

$$\frac{d(GT)}{dt} = k_1 GT - k_2 G_{\mathrm{in}} T = 0 \qquad (10)$$

d'où

$$GT = \frac{k_1}{k_2} G_{\mathrm{in}} T \qquad (11)$$

substitué dans (9)

$$V_{\mathrm{in}} + k_2 G_{\mathrm{ex}} T - k_2 G_{\mathrm{in}} T = 0 \text{ ou } G_{\mathrm{in}} = G_{\mathrm{ex}} + \frac{V_{\mathrm{in}}}{k_2 T} \qquad (12)$$

Si on considère que G_{ex} est négligeable devant G_{in} et que

$$V_{\mathrm{in}} = V_{\mathrm{in}}^{\mathrm{max}} \frac{G_{\mathrm{ex}}}{K_m + G_{\mathrm{ex}}}$$

on a:

$$G_{\mathrm{in}} = \frac{V_{\mathrm{in}}^{\mathrm{max}}}{k_2 T} \frac{G_{\mathrm{ex}}}{K_m + G_{\mathrm{ex}}} \qquad (13)$$

formellement identique à l'équation (4), avec $k_{\mathrm{ex}} = k_2 T_{\mathrm{in}}$.

La constante de sortie est donc une constante de transport proportionnel au gradient dans la mesure où T_{in} est lui-même constant.

La constance de ce niveau de concentration de T doit être ajusté en régime stationnaire. Au début de l'équilibration T_{in} apparaît dans l'espace intracellulaire en quantité stoichiométrique avec G_{in}, en régime stationnaire par contre sa concentration doit être très inférieure à G_{in} pour expliquer la lenteur de la sortie en cas d'inhibition de la perméase.

En outre pour expliquer la stimulation de la sortie du TDG par TMG et la constance de k_{ex} pour un substrat donné il faut que T_{in} dépende plus de la nature du substrat que du degré de saturation de la perméase. Un des mécanismes possible d'un tel ajustement consiste à supposer que la quantité totale de T, la somme de $R \sim T$, GT et T libre est limitée.

Quand la perméase ne fonctionne pas, tout est sous la forme $R \sim T$.

En régime stationnaire $R \sim T$ est regénéré à partir de R et de T libre avec une vitesse égale à V_{in}

$$R \times T \times k_3 = V_{\mathrm{in}} \qquad (16)$$

Dans des conditions:

$$R = T + GT$$

or d'après (11) et (13)

$$GT = \frac{V_{\mathrm{in}}}{k_1}$$

substitué dans (16) on a

$$T\left(T + \frac{V_{\mathrm{in}}}{k_1}\right) = \frac{V_{\mathrm{in}}}{k_3}$$

si

$$\text{si } T \ll \frac{V_{\mathrm{in}}}{k_1}, \text{ ou } T \ll GT, \text{ on a: } T = \frac{k_1}{k_3}$$

T est ainsi constant pour un substrat donné quel que soit l'activité de la perméase, par contre il varie avec la nature du substrat puisque k_1, est la constante de vitesse de dissociation de *GT*. Si l'affinité de *T* est plus forte pour TDG que pour TMG, le niveau de *T* libre sera plus bas pour le premier que pour le second. Il s'ensuit que la vitesse de sortie de TDG sera accélérée en présence de TMG. Ce même mécanisme expliquerait la sortie lente en cas d'inhibition de la perméase (Fig. 6) car dans ce cas T_{in} n'est pas régénéré.

L'effet du glucose peut s'expliquer également si ce sucre entre dans la cellule par un mécanisme distinct par sa stéréospécificité, mais qui libère dans la cellule le même transporteur. Comme le glucose est consommé au fur et à mesure de son entrée, *T* se libère en excès par rapport à son niveau de régime stationnaire et contribue à la sortie des galactosides.

Ce dernier mécanisme fonctionne également en l'absence de galactoside-perméase, et réalise l'explusion active des galactosides par le glucose. C'est un exemple de transport actif peu spécifique des galactosides, couplé au transport actif enzymatique du glucose et du transporteur *T*.

Le nombre des faits à expliquer nous a obligé à élaborer un modèle aussi détaillé, il va sans dire que ce n'est qu'un modèle possible et que l'identification des constituants du système peut seul apporter la solution du mécanisme des perméases et peut être du transport actif en général.

RÉSUMÉ

L'accumulation de thiogalactosides radioactifs dans l'espace intracellulaire de *E. coli* possédant la galactoside-perméase aboutit à un état stationnaire. La mesure de la concentration intracellulaire, de l'affinité et de la vitesse d'échange permet de calculer l'activité du mécanisme d'entrée et la constante de vitesse de la sortie passive. Le mécanisme d'entrée est catalytique, il est couplé avec l'énergie métabolique. La consommation d'énergie correspond à l'hydrolyse d'une molécule d'ATP par molécule de galactoside transporté. La sortie passive n'est pas une diffusion libre, mais peut être interprété comme une diffusion facilitée en combinaison avec une molécule transporteur.

Le comportement des paramètres d'entrée et de sortie dans des conditions variées est expliqué par un modèle cohérent.

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LACTIC DEHYDROGENASE OF YEAST

III. A COMPARATIVE STUDY OF THE KINETIC PROPERTIES AND THE STABILITY OF TWO ISOLATED FORMS OF THE ENZYME

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(Received August 12th, 1959)

SUMMARY

The kinetic properties and the stability of two isolated fractions of yeast lactic dehydrogenase (LDH II and III) have been compared. LDH II oxidized L(+) and LDH III oxidized D(—) lactic acid.

LDH II reduced cytochrome b_2. LDH III contained a heme which was irreducible by LDH III as well as by LDH II.

Crude and purified LDH II were completely inactivated by incubation at 55–57° for 5 min and by low concentrations of *p*-chloromercuribenzoate (10^{-4} *M* or less). LDH III lost the ability to reduce ferricyanide and 2,6-dichlorophenol indophenol under the same conditions, but the reduction of cytochrome *c* was unaffected.

The reduction of cytochrome *c* by LDH II was not influenced by variations in the salt concentration. LDH III was either inhibited by the addition of small amounts of several salts, or its affinity for cytochrome *c* was greatly decreased.

The affinities for ferricyanide and 2,6-dichlorophenol indophenol appeared to be similar for both forms of the enzyme. The reactions with these dyes were unaffected by great changes in the salt concentration.

LDH II and III had rather flat pH optima with cytochrome *c* and with ferricyanide as acceptors. The regions of optimum pH were somewhat different for the two forms of the enzyme.

It is argued that the two forms of LDH are no artefacts; they must occur already in the yeast cell.

INTRODUCTION

In the previous papers of this series[1,2,3] the isolation of several forms of yeast lactic dehydrogenase (LHD) was described. These forms could be separated on a NN-diethylaminoethylcellulose exhanger and therefore differed in the negative charge available to the exchanger. Furthermore, the spectral properties, the flavin/heme ratio, and the activity were different in the various forms. In the present paper the kinetic properties and the stability of two forms of LDH will be described and compared.

MATERIALS AND METHODS

The following materials were used in addition to those described previously[2,3].

D(+) *lactic acid* (Ca. salt) was purchased from Mann Research Laboratories, N.Y. The specific rotation in molybdate solution was + 36.3°.

L(+) *lactic acid* was obtained from Mann Research Laboratories (40 % solution) and from California Foundation for Biochemical Research, Los Angeles (Ca. salt. $[\alpha]_D = -3.84$; 6.65 % in water at 15°).

Trypsin (twice crystallized) was purchased from Nutritional Biochemicals Corporation, Clevland, Ohio.

Desoxyribonuclease was obtained from L. Light & Co. Ltd., Colnbrook, England.

Relative activities

The ratio's between the turnover numbers with cytochrome *c* ($2 \cdot 10^{-5}\,M$), ferricyanide ($5 \cdot 10^{-4}\,M$), and 2,6-dichlorophenol indophenol ($6 \cdot 10^{-5}\,M = 1.2 \cdot 10^{-4}\,N$) will be called "relative activities" except where otherwise stated. The reactions were carried out at 23° in phosphate buffer, μ 0.01, in the presence of $M/1000$ Versene.

The reaction with ferricyanide was measured at 420 mμ where the extinction coefficient was $1.0 \cdot 10^3\,M^{-1}\,\mathrm{cm}^{-1}$.

NOMENCLATURE

The first step in the purification procedure from yeast extract was the precipitation of the enzyme with acetone[2]. This precipitate, dissolved in water, had a pH of 6.3 and will be called "crude LDH".

A number of fractions of LDH were eluted from the column[1,2,3] and pooled in three mixtures of eluates, indicated by I, II and III, according to the order of elution.

LDH I

The variants of the enzyme which were eluted between pH 7 and 6 with 0.04 M phosphate (group I of eluates) will be called LDH I. They contained about 0.5 flavin per heme and showed spectral properties deviating from those of the crystalline enzyme of APPLEBY AND MORTON[5].

LDH II

The variants of the enzyme which were eluted at pH 5.3 with 0.04 M phosphate and zero to 0.08 M NaCl (group II of eluates) will be called LDH II.

LDH II contained one flavin per heme and the purest preparation obtained had an equivalent weight of 100,000. The spectral properties were similar to those of the crystalline enzyme[5].

LDH III

LDH III was eluted at pH 5.3 with 0.04 M phosphate and 0.4 M NaCl[1,4] (group III of eluates).

RESULTS

Crude preparations of LDH

The properties of crude LDH varied from preparation to preparation: (1) The relative activities have been observed to vary from 0.2:1:1 to 0.8:1:1; (2) the concentration of cytochrome *c* at which half-maximal activity was attained (in phosphate μ 0.08, pH 7.1) varied from $0.4 \cdot 10^{-5}$ *M* to $2 \cdot 10^{-5}$ *M*; and (3) the fraction of lactic dehydrogenase activity which remained after incubation at 55–57° for 5 min varied from 5–20 %. The same fraction of the enzyme remained after incubation with high concentrations of *p*-chloromercuribenzoate. This fraction (heat-stable and pCMB-stable) will be called stable LDH. The properties of stable LDH were different from those of the original preparation. In the first place, the ratio of the reduction of cytochrome *c* to the reduction of the dyes was higher for the stable enzyme. The relative activities varied from 12:1:1 to 7:1:1. Secondly, the reaction of the stable enzyme with cytochrome *c* was strongly affected by changes in the salt concentration, whereas that of the original preparation was for the most part uninfluenced by the same conditions. In some preparations the activity of the stable enzyme decreased by about 80 % when the ionic strength of the phosphate buffer was increased from 0.01 to 0.08 (pH 7.1), in others the affinity of the enzyme for cytochrome *c* was greatly decreased.

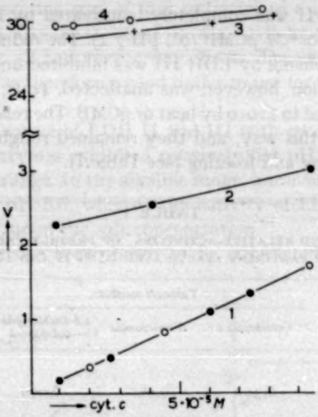

Fig. 1. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0.02 *M*) by a crude preparation of LDH (curves 3 and 4) and by the heat-stable (curves 1 and 2) and the pCMB-stable (curve 1, marked ○—○—○) enzyme contained in this preparation (see, the text). All solutions were at pH 7.1 and contained *M*/1000 Versene. Curve 1: phosphate μ 0.08; curve 2: phosphate μ 0.01; curve 3: phosphate μ 0.08; curve 4: phosphate μ 0.01.

Fig. 1 illustrates the reaction properties of a crude preparation which contained 10 % stable enzyme. The relative activities of the original preparation was 0.8:1:1 compared with 12:1:1 for the stable enzyme. It is seen from the figure that both the original preparation and the stable enzyme followed zero-order kinetics with cytochrome *c* as acceptor in the region 10^{-5}–10^{-4} *M* when the phosphate concentration was very low (μ 0.01). When the buffer concentration was increased to μ 0.08, however, the reaction properties of the original enzyme were practically unaltered, whereas the reaction of the stable enzyme changed from zero to first-order kinetics.

Incubation of the crude preparation at 37° for 30 min did not change the amount of stable enzyme.

A comparison of the properties of isolated LDH II and III

Yield, composition, and purity. In preparations which contained only small amounts of LDH I[3], LDH II constituted the main part of the LDH activity of crude preparations. The composition and purity of this form of the enzyme have been described previously[2,3].

When the DEAE-column was eluted by a gradual increase in the NaCl concentration from 0.08 to 0.4 *M*, an inactive haemoprotein was eluted with 0.4 *M* NaCl, which contained one mole of cytochrome b_2/100,000 g of protein. The haemoprotein was not associated with flavin. Between this fraction and LDH II cytochrome *c* peroxidase was eluted as a brown zone with one heme/200,000 g of protein. In order to obtain active LDH III, the salt concentration was increased stepwise from 0.08 to 0.4 *M* NaCl. In this way, preparations which contained 0.2 flavin/heme/500,000 g of protein were obtained. The heme was of type 2[3] and irreducible by LDH III as well as by LDH II. The amount of LDH III obtained corresponded roughly to the amount of stable enzyme in the crude preparation.

The stability. LDH II was completely inactivated by incubation at 55–57° for 3 min (pH 7.1) and by 10^{-4} *M* pCMB (0°, pH 7.1). The reduction of ferricyanide and 2,6-dichlorophenol indophenol by LDH III was inhibited under the same conditions; the cytochrome *c* reduction, however, was unaffected. Thus, the relative activities of LDH III could be changed to 1:0:0 by heat or pCMB. The relative activities of LDH II could not be altered in this way, and they remained roughly the same also during inactivation due to ageing and freezing (see Table I).

TABLE I

ABSOLUTE AND RELATIVE ACTIVITIES* OF FRESHLY PREPARED AND OF PARTIALLY INACTIVATED LDH II AND III

Treatment	Turnover numbers			Relative activities
	Cytochrome c	Ferricyanide	2,6-Dichlorophenol indophenol	
II Freshly prepared	6,300	10,000	6,000	1.0:1.7:1.0
II Aged at 6° for 70 h	3,300	5,500	3,000	1.1:1.8:1.0
II Frozen, thawed	2,000	2,900	2,000	1.0:1.5:1.0
II Stored at — 10° for 3 weeks	120	240	120	1.0:2.0:1.0
II Trypsin-digested	180	400	—	1.0:2.2 —
III Freshly prepared	6,000	210	180	15:1.2:1.0
III Trypsin-digested	420**	40	40	10:1:1
III Venom-digested	480**	40	40	10:1:1

*The assays were carried out in phosphate μ 0.1 + *M*/1000 Versene, pH 7.1 LDH III was determined with 10^{-4} *M* cytochrome *c*.

**The rate was proportional to the cytochrome *c* concentration at least up to 10^{-4} *M* cytochrome *c*, just as for the original enzyme.

The reduction of cytochrome *c* by LDH III was inhibited within one minute at 100°. In $3 \cdot 10^{-3}$ *M* pCMB 20 % of the activity was destroyed within 5 h at 0° (pH 7.1).

The reaction with cytochrome c. Neither LDH II nor LDH III had constant kinetic properties. The concentration of cytochrome *c* at which half-maximal activity was attained varied from preparation to preparation. The sensitivity to salts was, however, widely different for LDH II and III. The cytochrome *c* dependency curve for LDH II and its insensitivity to salts was similar to that of the special crude preparation from which it was obtained, and the cytochrome *c* dependency curve for LDH III and its sensitivity to salt was similar to the stable enzyme of the crude preparation.

In general, LDH II followed zero-order kinetics down to a concentration of 10^{-5} *M* cytochrome *c*, and the rate was the same in phosphate μ 0.01, 0.08, and 0.08 + 0.04 *M* NaCl. LDH III, on the other hand, was either strongly inhibited by salts, or the affinity for cytochrome *c* was strongly decreased by changes in the salt concentration. Fig. 2 shows the reaction properties of LDH III in three different solutions. In phosphate, μ 0.01, the enzyme followed zero-order kinetics at least down to 10^{-5} *M*; in phosphate, μ 0.08, the reaction was of first order up to a concentration of 10^{-4} *M* cytochrome *c*. Fig. 3 illustrates the fact that the same salt dependency exists at pH 7.9. NaCl, Na_2SO_4, and NaAc all had very strong effects. The effect of glycylglycine was much smaller, and this was therefore a good buffer to use for the determination of pH dependency.

The pH-dependency curve of LDH II and III with cytochrome *c* as acceptor is shown in Fig. 4. Both enzymes exhibited relatively flat pH optima, and the curves were similar in the acidic range. In the alkaline range, however, the activity of LDH II decreased rapidly above pH 8.6, whereas the activity of LDH III decreased above pH 7.6 or 7.9, depending upon the salt concentration.

LDH II has previously[2] been shown to be stimulated to varying degrees by

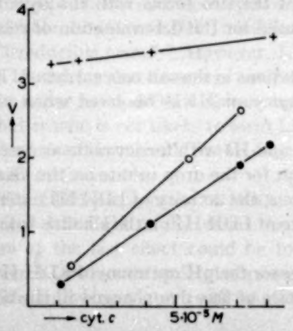

Fig. 2. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (D, 0.02 *M*) by a preparation of LDH III obtained from the crude preparation described in Fig. 1. All solutions were at pH 7.1 and contained *M*/1000 Versene. ●—●—● phosphate μ 0.08 + 0.04 *M* NaCl; ○—○—○ phosphate μ 0.08; +—+—+ phosphate μ 0.01.

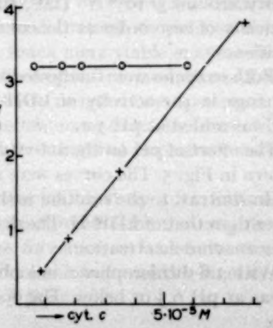

Fig. 3. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0.02 *M*) by a preparation of LDH III (the same as in Fig. 2). Both solutions were at pH 7.9. ○—○ glycylglycine 0.005 *M*; +—+ glycylglycine 0.005 *M* + 0.04 *M* NaCl.

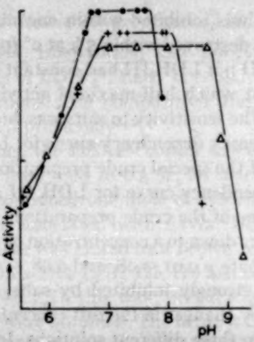

Fig. 4. The rate of the reduction of cytochrome *c* ($2 \cdot 10^{-5}$ *M*) by LDH II and III as a function of pH (DL lactate, 0.02 *M*). ●—● LDH III in 0.002 *M* phosphate + 0.002 *M* glycylglycine; +—+ LDH III in phosphate μ 0.08 + 0.002 *M* glycylglycine + *M*/1000 Versene; △—△ LDH II in phosphate μ 0.08 + *M*/1000 Versene, below pH 7.7; in glycylglycine, 0.05 *M*, from pH 7.7 to 8.6; in glycine, 0.05 *M*, from pH 8.6 to 9.7. Change of buffer at pH 8.6 and 7.7 did not affect the activity.

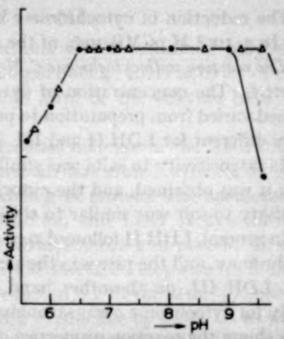

Fig. 5. The rate of the reduction of ferricyanide ($5 \cdot 10^{-4}$ *M*) by LDH II and III as a function of pH (DL lactate 0.02 *M*). ●—● LDH II; △—△ LDH III. Buffers: phosphate μ 0.08 at pH 7.7 and below; glycylglycine, 0.05 *M*, from pH 7.6 to 8.6; glycine, 0.05 *M*, from pH 8.6 to 9.7. Change of buffer at pH 8.6 and 7.7 did not affect the activity.

phenazin methosulfate. This compound had no effect on the preparations of LDH III thus far investigated.

The reaction with ferricyanide and 2,6-dichlorophenol. The reaction of LDH II and III with ferricyanide as acceptor was of zero order at least down to a concentration of $2 \cdot 10^{-5}$ *M*, and the reaction with 2,6-dichlorophenol indophenol attained half-maximal velocity around $3 \cdot 10^{-5}$ *N*. Thus, the reaction of the two forms with the dyes were practically of zero order at the concentrations used for the determination of relative activities.

Both enzymes were unaffected by great variations in the salt concentration. Thus, no change in the activity of LDH III with ferricyanide was observed when 0.8 *M* NaCl was added at pH 7.1.

The effect of pH on the activity of LDH II and III with ferricyanide as acceptor is shown in Fig. 5. The curves were similar except for the drop in rate on the alkaline side. In contrast to the reaction with cytochrome *c*, the activity of LDH III extended further than that of LDH II. The drop in the rate of LDH III on the alkaline side was due to enzyme inactivation.

With 2,6-dichlorophenol indophenol as acceptor the pH optimum of LDH II and III was at pH 6.5 or below. The optical properties of this dye changed in the acidic range.

Absolute and relative activities. The most active preparation of LDH II now obtained had relative activities 1.0:1.0:1.0 and turnover numbers 15,000 min^{-1} (per mole of flavin or heme) with cytochrome *c*. Both the absolute and the relative activities varied greatly[2,3]. In crude preparations which contained only small amounts of LDH I, the relative activities of LDH II were similar to those of the original preparation.

The first-order kinetic constant of a preparation of LDH III in phosphate μ 0.08, pH 7.1, has primarily been estimated at $1 \cdot 10^{6}\ M^{-1}\ \text{sec}^{-1}$. The constant was determined on the basis of flavin content. The relative activities of LDH III varied from preparation to preparation. The relative reduction of cytochrome *c* was much higher than for LDH II, and the relative activities were similar to those of the heat- and pCMB-stable enzyme of the crude preparation.

The effect of decomposing enzymes. Attempts were made to alter the kinetic properties of LDH II and III by means of degrading enzymes. Trypsin inactivated both LDH II and III (see Table I), but the relative activities remained the same during inactivation, and the partially inactivated LDH III followed first-order kinetics with cytochrome *c* (from 10^{-5} to $10^{-4}\ M$) in phosphate μ 0.08, pH 7.1, just as did the original enzyme. The same was true for a preparation of LDH III that had been 80% inactivated by the venom of *Crotalus terrificus terrificus*.

Although LDH II as well as LDH III appeared to be associated with polynucleotides[1,4,6], neither ribonuclease nor desoxyribonuclease affected the kinetic properties of the two forms of the enzyme.

Stereospecificity. The stereospecificity of LDH II and III has been investigated in phosphate μ 0.01, pH 7.1, and with $2 \cdot 10^{-5}\ M$ cytochrome *c* as acceptor LDH II was found to be specific for L(+) and LDH III for D(—) lactic acid.

DISCUSSION

The data obtained show clearly that both L(+) and D(—) lactic dehydrogenase are present in yeast extracts. The fact that neither LDH II nor LDH III had constant kinetic properties indicates that decomposition may take place during the extraction procedure. This may account for the fact that LDH with differing reaction properties has been obtained by other authors as well[7,8,9].

If only one form of the enzyme is present in the cell, LDH II must have changed its stereospecificity and decomposed to yield LDH III, or vice versa. This seems improbable, however. LDH II has been observed to decompose to yield an enzyme with irreducible heme[2,3]. However, LDH III is a much more stable enzyme than is LDH II and for that reason is not likely to be a decomposition product of LDH II. On the other hand, LDH III does not have the ability to reduce cytochrome b_2 and for that reason is not likely to form LDH II. Therefore, more than one form of LDH may be present in the cell. In this connection it is interesting that respiratory enzymes in yeast are inducible.

The LDH-preparation of BOERI AND TOSI[10] was inhibited by salts. Salts either inhibited LDH III or decreased the affinity of cytochrome *c* for the enzyme. The nature of the last effect could be to prevent the formation of a definite complex between LDH-lactate and cytochrome *c*. This would result in first-order kinetics.

The following sequence of reactions could take place:

1. $\text{E} + \text{lactate} \rightleftarrows \text{E. lactate}$
2. $\text{E. lactate} + \text{cytochrome } c^{\cdot\cdot\cdot} \rightarrow \text{E} + \text{pyruvate} + \text{cytochrome } c^{\cdot\cdot}$

For LDH II the reduction of cytochrome *c* as well as the reduction of the dyes are sensitive to pCMB. For LDH III, however, exposed sulfhydryl groups appear to be essential for the reduction of the dyes only.

In conclusion, the salt effects as well as the effect of pCMB indicate that one difference between LDH II and III is in the reaction with cytochrome *c*, where two different attachment sites may be operating.

ACKNOWLEDGEMENT

The author is indebted to Norges Almenvitenskapelige Forskningsråd for financial support and to Mr. ROBERT TEISTÖL for technical assistance.

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THE ACTION OF MAMMALIAN LIVER ENZYME PREPARATIONS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

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(Received August 18th, 1959)

SUMMARY

α-N-alkyl derivatives of DL-asparagine were not hydrolyzed by rat-liver asparaginase or guinea pig-liver asparaginase. They markedly inhibited the action of these enzymes on L-asparagine. In all cases tested the inhibition was found to be competitive. Rat and guinea pig liver asparaginase did not hydrolyze N-(β-DL-aspartyl)-alkylamines and were inhibited by these compounds only slightly, if at all. The asparagine-α-keto acid transamination-deamidation system did not act on both kinds of asparagine derivatives and was not inhibited by them. Rat-liver preparations which exhibited asparaginase activity also catalyzed β-aspartyl transfer from asparagine to hydroxylamine. The specificity of the transfer reaction towards both kinds of asparagine derivatives was the same as in the case of asparaginase.

INTRODUCTION

Asparaginase is widely distributed in animal and plant tissues, and in microorganisms[1]. A comprehensive study of the specificity of asparaginase activity was carried out by

Grassmann and Mayr[2] with asparaginase prepared from brewer's yeast. They found that this enzyme acted on asparagine only when its amino and amido groups were free. Working with rat-liver asparaginase, Greenstein and Price[3] found that this enzyme did not attack β-L-aspartyl-L-alanine and N-carbobenzoxy-L-asparagine. Similarly, Meister *et al.* found that DL-α-N-methylasparagine was not hydrolyzed by rat-liver asparaginase[4].

When rat-liver homogenates are heated at 50° for 10 min, they hydrolyze asparagine only in the presence of α-keto acids such as pyruvate[3,5]. This reaction is due to the presence in the homogenates of an asparagine-α-keto acid transamination-deamidation system. In this system a transamination between asparagine and α-keto acid is followed by the deamidation of the α-ketosuccinamic acid formed[6-8].

The present paper deals with the action of rat-liver and guinea pig asparaginase and of the rat-liver asparagine-α-keto acid transamination-deamidation system on α-N-alkyl derivatives of asparagine and on N-(β-aspartyl)-alkylamines. The effect of both kinds of asparagine derivatives on the above-mentioned enzymes was also investigated. In addition, the specificity of the β-aspartyl transfer reaction catalyzed by rat-liver preparations was studied.

EXPERIMENTAL

Materials and methods

L- and D-aspartic acid and L- and D-asparagine were purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio.

α-N-alkyl-derivatives of DL-asparagine

α-DL-chloro-β-succinamide was prepared from DL-asparagine according to the method of Holmberg[9]. It was crystallized from hot water. Found: Cl, 23.2; neutral equivalent, 150. Calculated: Cl, 23.4; neutral equivalent, 152. For amination, 1 mole of α-DL-chloro-β-succinamide was treated with 5 moles of alkylamines (33 % aqueous solution) for 3–4 days at 30°. The solutions were evaporated under reduced pressure at a temperature below 50° with repeated addition of water. The residues were treated in one of the following ways: (a) (For the methyl, ethyl, ethanol and isopropyl derivatives.) The residue was rubbed up with alcohol and kept in the cold. The precipitate was filtered, washed with alcohol and dried. The methyl and ethyl derivatives so obtained were twice recrystallized from water–alcohol. The ethanol derivative was crystallized from water–alcohol and then from hot 75 % alcohol. The isopropyl derivative was crystallized from water–acetone and afterwards from hot 90 % alcohol. (b) The propyl and butyl derivatives were obtained by dissolving the residue in a minimum amount of water and precipitating with 5 volumes of acetone. The products so obtained were recrystallized several times from water–acetone.

For analysis, the compounds were dried *in vacuo* at 100° over phosphorus pentoxide. DL-α-N-methylasparagine: Found: N, 19.0 %; neutral equivalent (Linderstrøm-Lang titration), 147. Calculated: N, 19.1; neutral equivalent, 146. DL-α-N-ethylasparagine: Found: N, 17.5; neutral equivalent (Linderstrøm-Lang titration), 160. Calculated: N, 17.5; neutral equivalent, 160. DL-α-N-propylasparagine: Found: N, 16.0; neutral equivalent (Linderstrøm-Lang titration), 172. Calculated: N, 16.1; neutral equivalent, 174. DL-α-N-isopropylasparagine: Found: N, 16.0; neutral equivalent (LINDERSTRØM-LANG titration), 174. Calculated: N, 16.1; neutral equivalent, 174. DL-α-N-butylasparagine: Found: N, 14.7; neutral equivalent (LINDERSTRØM-LANG titration), 185. Calculated: N, 14.8; neutral equivalent, 188. DL-α-N-ethanolasparagine: Found: N, 15.8; neutral equivalent (LINDERSTRØM-LANG titration), 171. Calculated: N, 15.9; neutral equivalent, 175.

N-(β-DL-aspartyl)-alkylamines

β-methyl ester hydrochloride of DL-aspartic acid was prepared according to the method of COLEMAN[10] by treating DL-aspartic acid with methanolic hydrochloric acid and precipitating the ester hydrochloride with ether. The methanolic hydrochloric acid was obtained from acetyl chloride and dry methanol as described by HANBY *et al.*[11]. The ester was converted into N-(β-DL-aspartyl)-alkylamines by treatment with 7 moles of the corresponding amine in 30 % alcoholic solutions at 35° for 5–6 days. The reaction mixtures were evaporated under reduced pressure. The methyl and 2-ethanol derivatives were obtained by dissolving the residues in water and precipitating with alcohol. The products were recrystallized from water–alcohol. The ethyl- and 3-propanol derivatives were obtained by rubbing up the residues with alcohol. The precipitates formed were recrystallized from hot water.

For analysis, the substances were dried *in vacuo* at 100° over phosphorus pentoxide. N-(β-DL-aspartyl)-methylamine: Found: N, 18.9; neutral equivalent (LINDERSTRØM-LANG titration), 144. Calculated: N, 19.1; neutral equivalent, 146. N-(β-DL-aspartyl)-ethylamine: Found: N, 17.5; neutral equivalent (LINDERSTRØM-LANG titration), 156. Calculated: N, 17.5; neutral equivalent, 160. N-(β-DL-aspartyl)-aminoethanol: Found: N, 15.9; neutral equivalent (LINDERSTRØM-LANG titration), 173. Calculated: N, 15.9; neutral equivalent, 175. N-(β-DL-aspartyl)-3-aminopropanol: Found: N, 15.0; neutral equivalent (LINDERSTRØM-LANG titration), 186. Calculated: N, 14.8; neutral equivalent, 189.

β-aspartohydroxamic acid was prepared as described elsewhere[16].

Rat liver enzyme

Adult albino rats (male or females) were killed by decapitation. The liver was rapidly removed, cut in small pieces, and homogenized in an M.S.E. blender (14,000 rev./min) in 4–5 volumes of cold water (0–2°) for 2 min. The pH of the homogenate was adjusted to 7.5 with NaOH 0.1 *N* and the homogenate was centrifuged in the cold for 45 min at 32,000 × *g*. The reddish transparent supernatant was used for enzymic experiments. For the experiments with the asparagine-α-keto acid transamination-deamidation system, the homogenate was kept 10 min at 50° and rapidly cooled before centrifugation. The supernatants so obtained are referred to as "asparaginase-free rat-liver enzyme".

Guinea pig-liver asparaginase

Asparaginase from guinea pig liver was prepared as described by KREBS[12]. Equal amounts of the finely powdered preparations were added to each reaction mixture.

Assay procedures

Asparaginase activity: The reaction mixture contained per ml of 0.08 *M* phosphate buffer, pH 7.4, substrate, 6 μmoles (unless otherwise stated), rat-liver enzyme, 0.2 ml, or guinea pig-liver preparation, 2.5 mg. Incubations were carried out at 37° for 1 h. At the end of the incubation 3-ml samples were removed and heated for 3 min at 70°. (No loss of NH_3 occurred under these conditions.) To these samples 2 ml of borate buffer (pH 10.1)[13] and ten drops of tributylphosphate were added. The NH_3 was transferred by aeration at 50° for 15–20 min into dilute HCl and measured colorimetrically by Nessler's method. With N-(β-DL-aspartyl)-methyl- or ethylamine as substrate the aeration was carried out at 60° into standard acid, which was then titrated. In these cases, as with N-(β-DL-aspartyl)-2-aminoethanol and N-(β-DL-aspartyl)-3-aminopropanol, a chromatogram was run at the beginning and the end of the reaction (phenol 80 %, Whatman paper No. 1). Aspartic acid was quantitatively estimated by elution and colorimetric determination as described by MEYER[14].

Asparagine-α-keto acid transamination-deamidation system: The reaction mixtures were the same as those given in previous section, but contained, in addition, 12 μmoles sodium pyruvate/ml phosphate buffer. Ammonia and amines were estimated as above. In addition, chromatographic estimations of alanine were made. In these cases, the enzyme solutions were dialyzed in the cold against phosphate buffer for several hours prior to incubation. We found that the asparagine-α-keto acid transamination-deamidation system was not affected by the dialysis.

β-Aspartyl-transfer activity: Each reaction mixture contained per 1 ml of 0.0375 *M* Tris buffer, pH 7.2, substrate, 21 μmoles; rat-liver enzyme, 0.25 ml; NH_2OH, 105 μmoles.

After incubation at 37° for 1 h, 2-ml samples were removed and 1 ml of 2 *M* NH_2OH, pH 6.5, and 3 ml of $FeCl_3$-reagent[15] were added. After centrifugation the hydroxamic acid-ferric complex was estimated in a Klett-Summerson colorimeter (Filter 54) using synthetic β-aspartohydroxamic acid as standard.

RESULTS

Rat-liver and guinea pig-liver asparaginase failed to liberate ammonia or alkylamines from the following derivatives of asparagine: DL-α-N-methylasparagine; DL-α-N-ethylasparagine; DL-α-N-propylasparagine; DL-α-N-isopropylasparagine; DL-α-N-butylasparagine; DL-α-N-ethanolasparagine; N-(β-DL-aspartyl)-methylamine; N-(β-DL-aspartyl)-ethylamine. Under the same conditions, unsubstituted asparagine was readily hydrolyzed. The experiments were carried out at a pH range varying between 6 and 8. Methyl- or ethylamine added to the reaction mixture in amounts corresponding to those of the substrates gave on aeration a recovery of 75 % and 55 % respectively.

In the experiments with N-(β-DL-aspartyl)-2-aminoethanol or 3-aminopropanol at a pH range varying between 6 and 8 no cleavage occurred, since no formation of aspartic acid could be detected by quantitative paper chromatography. Asparagine as substrate in control experiments under the same conditions gave a fair correlation between ammonia as estimated by aeration, and aspartic acid estimated by chromatography.

α-N-alkyl derivatives of DL-asparagine inhibited the action of rat-liver and guinea pig-liver asparaginase, as can be seen from Table I. In all cases the inhibitions obtained with the guinea pig-liver asparaginase were smaller than those obtained with the rat-liver asparaginase under similar conditions. The methyl, ethyl, propyl and butyl derivatives inhibited approximately to the same extent, whereas the inhibitions shown by the isopropyl or the ethanol derivatives were considerably lower. No inhibitions were obtained with the N-(β-DL-aspartyl)-alkylamines, with the exception of a small inhibition obtained with N-(β-DL-aspartyl)-methylamine in the case of rat liver asparaginase in a molar ratio inhibitor–substrate 10:1.

TABLE I

EFFECT OF ASPARAGINE DERIVATIVES ON RAT-LIVER AND GUINEA PIG-LIVER ASPARAGINASE

Reaction mixtures, as described under *Assay procedures.*

Asparagine derivative added, µmoles/ml		*Rat-liver asparaginase*		*Guinea pig-liver asparaginase*	
		NH_3 liberated µmoles*	*Inhibition %*	*NH_3 liberated* µmoles*	*Inhibition %*
None		6.05		4.20	
DL-α-N-methylasparagine	6	4.15	30		
DL-α-N-methylasparagine	12	3.05	50		
DL-α-N-ethylasparagine	6	4.05	33		
DL-α-N-ethylasparagine	12	3.00	52		
DL-α-N-ethylasparagine	18	2.05	65	3.25	23
DL-α-N-ethylasparagine	60	1.35	78	2.30	45
DL-α-N-propylasparagine	6	4.15	30		
DL-α-N-propylasparagine	18	2.40	60	3.20	25
DL-α-N-propylasparagine	60			2.10	50
DL-α-N-butylasparagine	6	4.00	35		
DL-α-N-butylasparagine	18	2.00	67	3.00	28
DL-α-N-butylasparagine	60			2.05	51
DL-α-N-isopropylasparagine	6	5.15	15		
DL-α-N-isopropylasparagine	18	4.10	32	3.70	12
DL-α-N-isopropylasparagine	60			2.85	32
DL-α-N-ethanolasparagine	6	4.85	20		
DL-α-N-ethanolasparagine	18	3.75	38	3.50	17
N-(β-DL-aspartyl)-methylamine	30	5.40	10	4.25	0
N-(β-DL-aspartyl)-methylamine	60	5.05	17		
N-(β-DL-aspartyl)-ethylamine	60	5.85	< 5	4.20	0
N-(β-DL-aspartyl)-2-aminoethanol	60	5.95	0		

* All blanks substracted.

Experiments carried out with DL-α-N-methyl- and ethylasparagine proved that the inhibition shown by these compounds is competitive (Figs. 1 and 2).

Asparaginase-free rat-liver enzyme, which in the presence of pyruvate was very active towards DL-asparagine, failed to liberate NH_3 from the α-N-alkyl derivatives of DL-asparagine. On incubation of rat-liver enzyme (free of asparaginase) in the presence of pyruvate with N-(β-DL-aspartyl)-methyl- or ethylamine, no liberation of methyl- or ethylamine could be detected. Similarly no formation of alanine could be observed by paper chromatography. In control experiments, asparaginase-free rat-liver enzyme was incubated in the presence of pyruvate with alanine in concentration as small as 5 % of those of the derivatives used in the experiments. The recovery of alanine in these control experiments was nearly quantitative. After incubations of the asparaginase-free rat-liver enzyme with DL-asparagine under similar conditions, a formation of alanine was observed in amount of about 80 % of the amount of NH_3 liberated.

Rat-liver enzyme preparations also hydrolyzed α-asparagine (isoasparagine), but this activity was only slightly affected on heating at 50° for 10 min, which led to the complete abolition of the action on asparagine. The hydrolysis of α-asparagine was not inhibited by the α-N-alkyl derivatives of DL-asparagine in a molar ratio of derivative to α-asparagine up to 10:1.

Rat-liver enzyme catalyses the transfer of the β-aspartyl group from asparagine

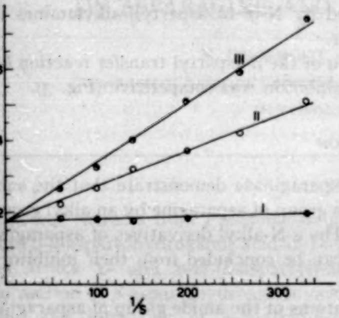

Fig. 1. Competitive inhibition of rat-liver asparaginase by DL-α-N-methyl- and DL-α-N-ethylasparagine. I: DL-asparagine; II: DL-asparagine and DL-α-N-methylasparagine (0.009 *M*); III: DL-asparagine and DL-α-N-ethylasparagine (0.018 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles ammonia/h.

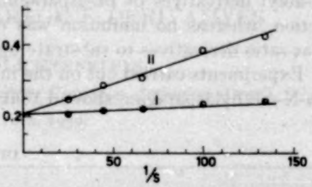

Fig. 2. Competitive inhibition of guinea pig-liver asparaginase by DL-α-N-ethylasparagine. I: DL-asparagine; II: DL-asparagine and DL-α-N-ethylasparagine (0.09 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles ammonia/h.

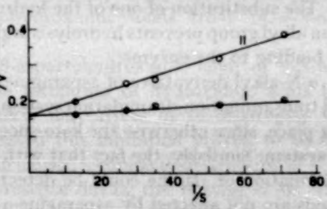

Fig. 3. Competitive inhibition of rat liver β-aspartyl transferase by DL-α-N-methylasparagine. I: DL-asparagine; II: DL-asparagine and DL-α-N-methylasparagine (0.021 *M*). *S* is the molar concentration of DL-asparagine. Velocity, *V*, is expressed in μmoles hydroxamic acid/h.

TABLE II

EFFECT OF ASPARAGINE DERIVATIVES ON THE β-ASPARTYL TRANSFER ACTIVITY OF RAT-LIVER ENZYME

Reaction mixtures, as described under *Assay procedures*.

Inhibitor, μmoles/ml		*Hydroxamic acid formed μmoles**	*Inhibition %*
None		5.40	
DL-α-N-methylasparagine	36	2.90	46
DL-α-N-methylasparagine	63	1.35	75
DL-α-N-ethylasparagine	36	3.15	42
DL-α-N-ethylasparagine	63	1.60	70
DL-α-N-propylasparagine	36	2.85	47
DL-α-N-propylasparagine	63	1.20	78
DL-α-N-butylasparagine	36	2.80	48
DL-α-N-butylasparagine	63	1.35	75
DL-α-N-isopropylasparagine	36	4.30	20
DL-α-N-isopropylasparagine	63	3.35	38
DL-α-N-ethanolasparagine	36	4.10	24
DL-α-ethanolasparagine	63	3.15	42
N-(β-DL-aspartyl)-methylamine	105	5.10	5
N-(β-DL-aspartyl)-ethylamine	105	5.50	0

* All blanks subtracted.

to hydroxylamine. Homogenates heated for 10 min at 50° completely lost this aspartyl transfer activity. No hydroxamic acid was formed by the rat-liver enzyme from α-N-alkyl derivatives of DL-asparagine or from N-(β-DL-aspartyl)-alkylamines. Only the L-form of asparagine reacted with hydroxylamine. Table II shows that α-N-alkyl derivatives of DL-asparagine markedly inhibited the β-aspartyl transfer reaction, whereas no inhibition was obtained by N-(β-DL-aspartyl)-alkylamines in molar ratio derivatives to substrate up to 6:1.

Experiments carried out on the inhibition of the β-aspartyl transfer reaction by DL-α-N-methylasparagine showed that this inhibition was competitive (Fig. 3).

DISCUSSION

Experiments with rat-liver and guinea pig asparaginase demonstrate that the substitution of one hydrogen atom of the α-amino group of asparagine by an alkyl group prevents the hydrolysis of the amide group. The α-N-alkyl derivatives of asparagine can, however, combine with the enzyme as can be concluded from their inhibitory action on asparagine hydrolysis.

The substitution of one of the hydrogen atoms of the amide group of asparagine by an alkyl group prevents hydrolysis and also prevents, or at least strongly diminishes, the binding to the enzyme.

α-N-alkyl derivatives of asparagine gave no ammonia with the asparagine-α-keto acid transamination-deamidation system. This proved that no transamination reaction took place, since otherwise the keto-succinamic acid formed would be deamidated by the system. Similarly, the fact that with the N-(β-aspartyl)-alkylamines as substrates no formation of alanine could be detected in this system, indicated that such compounds are not affected by asparagine-α-keto acid transaminase.

The similarity of the specificities of the asparaginase and the β-aspartyl transferase activities of rat-liver preparations seems to indicate that both reactions are catalyzed by the same enzyme.

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THE ACTION OF *PSEUDOMONAS FLUORESCENS* EXTRACTS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

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(Received August 18th, 1959)

SUMMARY

Cell-free extracts from *Pseudomonas fluorescens* hydrolyzed L- and D-asparagine, L- and D-β-aspartohydroxamic acid and α-N-alkyl derivatives of DL-asparagine, but failed to attack L- and D-N-(β-aspartyl)-alkylamines. The extracts also catalyze the formation of β-aspartohydroxamic acid from L- or D-asparagine and hydroxylamine and the formation of α-N-alkyl-β-aspartohydroxamic acids from DL-α-N-alkyl derivatives of asparagine and hydroxylamine.

The hydrolysis of D-asparagine, of D-β-aspartohydroxamic acid, of α-N-alkyl derivatives of DL-asparagine as well as the formation of hydroxamic acids from hydroxylamine and D-asparagine or α-N-alkyl derivatives of DL-asparagine were inhibited by α-amino acids. In the cases tested this inhibition proved to be competitive. Heating the extracts at 55° for 8 min caused a complete destruction of all the enzymic activities mentioned above. The presence during this heating of one of the substrates or of one of the amino acids that caused inhibition gave a complete or partial protection of all the above mentioned enzymic activities.

The question whether the enzymic activities mentioned are catalyzed by one or more enzymes is discussed.

INTRODUCTION

In a previous communication the specificity of asparaginase and of β-aspartyl transferase activities of mammalian-liver preparations were studied with the aid of asparagine derivatives bearing various alkyl residues in the amino or in the amide groups[1]. The present paper deals with the specificity of these enzymic activities of bacterial origin (*Pseudomonas fluorescens*), using the same asparagine derivatives.

MATERIALS AND METHODS

Materials

The amino acids were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

DL-α-N-methyl-, ethyl-, propyl-, butyl-, isopropyl-, ethanolasparagine, N-(β-DL-aspartyl)-methylamine, -ethylamine, -2-aminoethanol, -3-aminopropanol and L- and D-β-aspartohydroxamic acid were prepared as described elsewhere[1-2].

N-(β-DL-aspartyl)-allylamine, N-(β-L-aspartyl)- ethylamine and N-(β-D-aspartyl)-ethylamine were prepared in a way similar to that described for N-(β-DL-aspartyl)-ethylamine. Analyses: N-(β-DL-aspartyl)-allylalamine: Found: N, 16.4; neutral equivalent (LINDERSTRØM-LANG titration), 168. Calculated: N, 16.3; neutral equivalent, 172. N-(β-L-aspartyl)-ethylamine: Found, N, 17.5; neutral equivalent (LINDERSTRØM-LANG titration), 160. Calculated: N, 17.5; neutral equivalent, 160. N-(β-D-aspartyl)-ethylamine: Found: N, 17.4; neutral equivalent (LINDERSTRØM-LANG titration), 158. Calculated: N, 17.5; neutral equivalent, 160.

DL-N-ethylaspartic acid was prepared in the following way: 10 g DL-α-N-ethyl-asparagine were heated for 8 h with 100 ml of hydrochloric acid 1:1. The mixture was evaporated *in vacuo* to dryness with repeated addition of water. The residue was dissolved in 40 ml of water, the pH was adjusted to 3.1 with lithium hydroxyde 2.5 *N* and the *N*-ethylaspartic acid was precipitated by adding 8 volumes of alcohol. The product was crystallized from water–alcohol. Analysis: Found: N, 8.6; neutral equivalent (LINDERSTRØM-LANG titration), 160. Calculated: N, 8.7; neutral equivalent, 161.

Enzyme preparation

The *Pseudomonas fluorescens* used in this study was obtained from the collection of the Department of Bacteriology of the Hebrew University-Hadassah Medical School. The bacteria were grown in Roux bottles at 30° for 40 h containing the following medium: agar, 25 g; peptone, 10 g; sodium chloride, 3 g; potassium dihydrogen phosphate, 3 g; magnesium sulfate (hydrate), 1 g/l of tap-water. The bacteria were harvested, centrifuged, washed twice with sodium chloride 0.9 % and dried *in vacuo* in a desiccator over calcium chloride. The finely powdered dry preparations preserve their activity for several months. Cell-free *Pseudomonas* extracts were prepared in the following way. A suspension of 70 mg of dried bacteria in 20 ml of water was held for 15 min in a 9 kc Raytheon sonic oscillator. After addition of 12 mg of L-asparagine, the mixture was kept for 10 min at 55°, dialyzed overnight against distilled water in the cold, and centrifuged at 15,000 × *g* for 10 min. The clear supernatant was used for enzymic experiments*.

Assay procedures

Hydrolysis of asparagine and its α-N-alkyl derivatives: The reaction mixture contained per ml of 0.045 *M* tris-(hydroxymethyl)-aminomethane buffer, pH 7.5, 6 µmoles of substrate and 0.1 ml of enzyme solution. After incubation for 1 h at 35°, 1 ml of 5 % trichloroacetic acid was added to 3 ml of incubation mixture. After addition of 3 ml of borate buffer pH 10.1[5] and some drops of tributyl phosphate, the ammonia was distilled into 0.1 *N* HCl by aeration at 50° for 10–15 min and estimated colorimetrically with the aid of Nessler's reagent.

Hydrolysis of L- *and* D-*β-aspartohydroxamic acid*: The reaction mixture contained per 1 ml of Tris buffer 0.045 *M*, pH 7.5, 4 µmoles of substrate and 0.1 ml of enzyme solution. After incubation for 1 h at 35°, 2-ml samples were withdrawn. To these samples 1 ml of NH_2OH 2 *M*, pH 6.5 and 3 ml of $FeCl_3$ reagent[6] were added. After centrifugation the hydroxamic acid was quantitatively measured in a Klett-Summerson colorimeter (filter 54).

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* The heat treatment is necessary to destroy the aspartase activity present in *Pseudomonas* extracts[3,4] which might obscure the results of L-asparagine hydrolysis. The presence of L-asparagine during the heating protects the other enzymic activities described in this paper (see p. 107).

β-aspartyl transfer

The reaction mixture contained per 1 ml of Tris buffer 0.030 *M*, pH 7.5, 30 µmoles substrate, 240 µmoles NH_2OH and 0.3 ml of enzyme solution. After incubation for 1 h at 35°, samples of 2 ml were removed and the hydroxamic acid determined as described above.

In several experiments aspartic acid was determined by quantitative paper chromatography. Chromatograms were run on Whatman paper No. 1 with phenol 80 % as solvent. The aspartic acid was eluted from the paper and estimated as described by Meyer[7].

RESULTS

Table I shows that *Pseudomonas* extracts catalyze the hydrolysis of L- and D-asparagine, L- and D-β-aspartohydroxamic acid and DL-α-N-alkyl derivatives of asparagine. They fail to attack N-(β-DL-aspartyl)-methyl- and ethylamine. Methyl and ethylamine added to the reaction mixture in amounts corresponding to those of the substrates gave on aeration recoveries of 75 % and 55 % respectively. Additional evidence for the stability of N-(β-aspartyl)-alkylamines towards the enzyme was obtained by chromatography, which failed to reveal the formation of aspartic acid. When L- or D-asparagine was incubated with the enzyme preparations under the same conditions, aspartic acid could be recovered chromatographically in amounts corresponding fairly well to the amounts of ammonia obtained by aeration.

DL-α-N-methyl-, ethyl-, and ethanolasparagine were hydrolyzed more rapidly than L- or D-asparagine. The rate of hydrolysis of the DL-α-N-alkyl derivatives of

TABLE I

THE ACTION OF *Pseudomonas* EXTRACTS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

Reaction mixtures, as given under *Assay procedures*.

Substrate	*Ammonia formed µmoles*	*Alkylamines liberated µmoles*	*Hydroxamic acid hydrolyzed µmoles*
L-asparagine	2.40		
L-asparagine*	2.40		
D-asparagine	2.25		
DL-asparagine*	2.40		
DL-α-N-methylasparagine	7.30		
DL-α-N-ethylasparagine	3.75		
DL-α-N-propylasparagine	1.45		
DL-α-N-butylasparagine	1.05		
DL-α-N-isopropylasparagine	0.25		
DL-α-N-ethanolasparagine	3.95		
N-(β-DL-aspartyl)-methylamine		0.00	
N-(β-L-aspartyl)-ethylamine		0.00	
N-(β-D-aspartyl)-ethylamine		0.00	
L-aspartohydroxamic acid			1.15
D-aspartohydroxamic acid			1.00

* Concentration, 0.012 *M*.

TABLE II

MAXIMUM HYDROLYSIS OF ASPARAGINE AND ITS α-N-ALKYL DERIVATIVES BY *Pseudomonas* EXTRACTS

Concentration of all substrates 0.0005 *M*. Buffer and enzyme as under *Assay Procedures*.

Substrate	*Ammonia formed, µmoles* *Time (h)*			*Maximum hydrolysis* %
	1	*2*	*3*	
L-asparagine	1.40	1.45	1.45	97
D-asparagine	1.50	1.53	1.53	102
DL-asparagine	1.35	1.42	1.42	95
DL-α-N-methylasparagine	0.72	0.73	0.73	48
DL-α-N-ethylasparagine	0.71	0.70	0.71	47

TABLE III

FORMATION OF HYDROXAMIC ACID FROM ASPARAGINE AND ASPARAGINE DERIVATIVES BY *Pseudomonas* EXTRACTS

Reaction mixtures, as under *Assay procedures*.

Substrate	*Hydroxamic acid formed, µmoles**
L-asparagine	2.50
D-asparagine	5.05
DL-α-N-methylasparagine	17.30
DL-α-N-ethylasparagine	7.30
DL-α-N-propylasparagine	3.60
DL-α-N-butylasparagine	3.05
DL-α-N-isopropylasparagine	0.65
DL-α-N-ethanolasparagine	7.60
N-(β-DL-aspartyl)-methylamine	0.00
N-(β-L-aspartyl)-ethylamine	0.00
N-(β-D-aspartyl)-ethylamine	0.00

* Synthetic aspartohydroxamic acid was used as standard in all cases.

asparagine markedly decreases from the methyl to the butyl derivatives. There is a striking difference in the susceptibility of DL-α-N-propylasparagine and DL-α-N-isopropylasparagine to the action of the enzyme. While the incubation of small amounts of L-, D- and DL-asparagine with the *Pseudomonas* extracts led to nearly 100% hydrolysis, the hydrolysis of DL-α-N-methyl- and ethylasparagine never exceeded 50% (Table II).

The cell-free extracts of *Pseudomonas* catalyze the transfer of the β-aspartyl group from L- and D-asparagine to hydroxylamine. The extracts are also capable of transferring the DL-α-N-alkyl-β-aspartyl groups from the corresponding DL-α-N-alkyl derivatives of asparagine to hydroxylamine, but fail to bring about the transfer of the β-aspartyl group from N-(β-aspartyl)-alkylamines to hydroxylamine. The results are summarized in Table III. Comparative quantitative evaluation was not possible because of the lack of synthetic α-N-alkyl-β-aspartohydroxamic acids.

On incubation of D-asparagine with cell-free *Pseudomonas* extracts and hydroxylamine the concentration of β-aspartohydroxamic acid at first increases linearly. Then the velocity of hydroxamic acid formation falls until the concentration of hydroxamic acid reaches a maximum value and remains constant. Using L-asparagine as substrate,

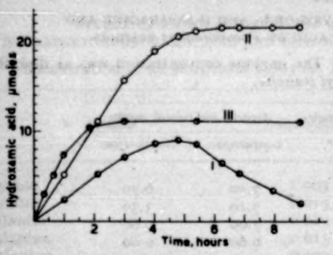

Fig. 1. Time-dependence curves of aspartotransferase activity towards L-asparagine (I), D-asparagine (II) and DL-α-N-ethylasparagine (III). Reaction mixtures as described under *Assay procedures*.

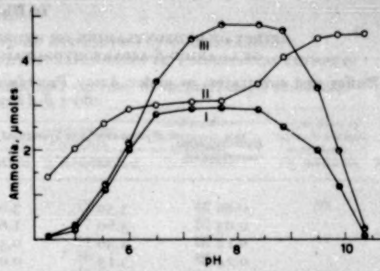

Fig. 2. pH-dependence curves for the hydrolysis of D-asparagine (I), of L-asparagine (II) and of DL-α-N-ethylasparagine (III). The buffers used were citrate–phosphate for pH 4.5 to 7.2 and borate for pH 7.5 o 10.4.

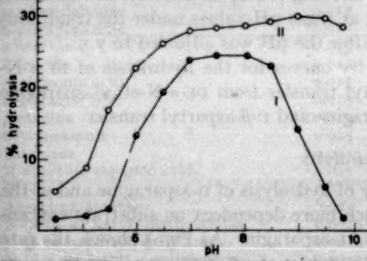

Fig. 3. pH-dependence curves for the hydrolysis of D-β-aspartohydroxamic acid (I) and L-β-aspartohydroxamic acid (II). The buffers used were citrate for pH 4.5 to 6.0, tris–maleate for pH 6.0 to 7.5 and borate for pH 7.5 to 10.0.

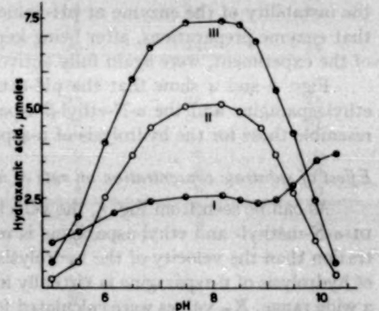

Fig. 4. pH-dependence curves for β-aspartyl transfer from L-asparagine (I) and from D-asparagine (II) to hydroxylamine and α-N-ethyl-β-aspartyl transfer from DL-α-N-ethylasparagine (III) to hydroxylamine. The buffers used were citrate for pH 4.5 to 6.0, Tris–maleate for pH 6.0 to 7.5 and borate for pH 7.5 to 10.0.

the concentration of aspartohydroxamic acid goes through a maximum and then falls to zero (Fig. 1). These results can be explained as follows. Hydroxylamine, in the concentration used in the experiments on β-aspartyl transfer, strongly inhibits the hydrolysis of D-asparagine and D-aspartohydroxamic acid by the extracts, but hardly affects the hydrolysis of L-asparagine and L-aspartohydroxamic acid (Table IV). It follows from Fig. 1, that the hydroxamic acid formed from DL-α-N-ethyl-asparagine was not hydrolyzed under the conditions of the experiments.

Effect of pH

As can be seen from Figs. 2, 3 and 4, the pH-activity curves for the hydrolysis of L-asparagine and L-β-aspartohydroxamic acid and for the L-β-aspartyl transfer are quite similar. There is also a marked similarity between the pH-activity curves for the hydrolysis of D-asparagine and D-aspartohydroxamic acid and for the β-D-aspartyl transfer. There are significant differences between the two groups of pH-activity

TABLE IV

EFFECT OF HYDROXYLAMINE ON HYDROLYSIS OF L- AND D-ASPARAGINE AND OF L- AND D-β-ASPARTOHYDROXAMIC ACID BY *Pseudomonas* extracts

Buffer and substrates, as under *Assay Procedures*. The enzyme concentration was as described under *β-Aspartyl transfer*.

Hydroxylamine concentration	*Hydroxamic acid hydrolysed, μmoles*		*Aspartic acid formed, μmoles*	
	L-*AHA**	D-*AHA**	L-*asparagine*	D-*asparagine*
0.00 *M*	3.50	3.05	7.20	6.70
0.03 *M*	3.60	1.60	7.10	3.50
0.12 *M*	3.50	0.30	7.00	0.60
0.24 *M*	3.15	0.00	6.60	0.00

* The abbreviation AHA is used for aspartohydroxamic acid.

curves at pH-values below 6 and especially above 9. These differences are not due to the instability of the enzyme at pH-values showing a drop in activity. It was found that enzyme preparations, after being kept at these pH-values under the conditions of the experiment, were again fully active after the pH was adjusted to 7.5.

Figs. 2 and 4 show that the pH-activity curves for the hydrolysis of DL-α-N-ethylasparagine and the α-N-ethyl-β-aspartyl transfer from DL-α-N-ethylasparagine resemble those for the hydrolysis of D-asparagine and D-β-aspartyl transfer.

Effect of substrate concentration on rate of hydrolysis

As can be seen from Fig. 5, the velocity of hydrolysis of D-asparagine and of the DL-α-N-methyl- and ethyl-asparagine is much more dependent on substrate concentration than the velocity of the hydrolysis of L-asparagine. As Fig. 5 shows, the rate of hydrolysis of L-asparagine is virtually independent of substrate concentration over a wide range. K_m values were calculated for D-asparagine, DL-α-N-methyl- and ethyl-asparagine from the slope of the $1/S$ against $1/V$ lines and the points of interception of these lines with the ordinate. The results were: K_m D-asparagine $= 4.8 \cdot 10^{-4}$ mole/l; K_m DL-α-N-methylasparagine $= 7.0 \cdot 10^{-4}$ mole/l; K_m DL-α-N-ethylasparagine =

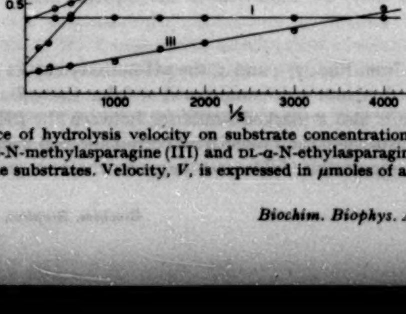

Fig. 5. The dependence of hydrolysis velocity on substrate concentration for L-asparagine (I), D-asparagine (II), DL-α-N-methylasparagine (III) and DL-α-N-ethylasparagine (IV). S is the molar concentration of the substrates. Velocity, V, is expressed in μmoles of ammonia formed/h.

TABLE V

EFFECT OF AMINO ACIDS ON THE RATE OF HYDROLYSIS OF D-ASPARAGINE, D-β-ASPARTOHYDROXAMIC ACID AND DL-α-N-METHYLASPARAGINE BY *Pseudomonas* EXTRACTS

Concentration of all substrates 0.005 *M*. Buffer and enzyme, as under *Assay Procedures*.

Inhibitor	*Concentration*	*D-asparagine hydrolysis, % inhibition*	*D-β-asparto-hydroxamic acid hydrolysis. % inhibition*	*Hydrolysis of DL-α-N-methyl-asparagine, % inhibition*
Glycine	0.005	45	55	50
Glycine	0.015	80	85	
L-alanine	0.005	54	50	61
L-alanine	0.015	88	90	
D-alanine	0.005	23	25	30
D-alanine	0.015	50	55	
L-serine	0.005	48	56	55
L-serine	0.015	80	90	
D-serine	0.015	44	52	50
D-aspartic acid	0.005	32	36	35
L-aspartic acid	0.005	78	85	85
D-phenylalanine	0.015	10		
L-phenylalanine	0.015	20	25	25
D-leucine	0.015	12		
L-leucine	0.015	25		
D-glutamic acid	0.015	23	23	25
L-glutamic acid	0.015	53	60	55
β-alanine	0.020	20		
Glycineamide	0.025	0	0	0
Sarcosine	0.025	0	0	0
N-ethyl-DL-aspartic acid	0.025	0	0	0
Proline	0.015	0	0	0
Acetyl-DL-alanine	0.025	0	0	0
N-(β-DL-aspartyl)-methylamine	0.00125	52		
N-(β-DL-aspartyl)-ethylamine	0.00125	52		
N-(β-D-aspartyl)-2-aminoethanol	0.00125	51		
N-(β-DL-aspartyl)-3-aminopropanol	0.00125	50		
N-(β-DL-aspartyl)-allylamine	0.00125	53		
N-(β-L-aspartyl)-ethylamine	0.010	90	90	90
N-(β-D-aspartyl)-ethylamine	0.010	12	15	15

TABLE VI

EFFECT OF AMINO ACIDS ON THE FORMATION OF HYDROXAMIC ACID FROM D-ASPARAGINE AND DL-α-N-ETHYLASPARAGINE BY *Pseudomonas* EXTRACTS

Reaction mixtures, as given under *Assay procedures*.

Inhibitor	*Concentration (M)*	*Formation of hydroxamic acid*	
		From D-asparagine, % inhibition	*From DL-α-N-ethylasparagine, % inhibition*
L-alanine	0.045	65	74
L-alanine	0.050	90	90
D-alanine	0.045	30	35
L-serine	0.045	68	78
D-serine	0.045	35	35
L-aspartic acid	0.030	80	90
D-aspartic acid	0.030	40	45
N-(β-L-aspartyl)-ethylamine	0.030	75	80
N-(β-D-aspartyl)-ethylamine	0.030	15	25

$3.2 \cdot 10^{-3}$ mole/l. The K_m value for L-asparagine could not be calculated in this way, but it is obviously much smaller than the K_m values for the other compounds.

Inhibition experiments

A number of α-amino acids tested inhibited the hydrolysis of D-asparagine, D-aspartohydroxamic acid, and the α-N-alkyl derivatives of DL-asparagine, and also inhibited the transfer reaction between D-asparagine or the α-N-alkyl derivatives of DL-asparagine and hydroxylamine. The inhibitory action of the L- and D-forms of several amino acids was compared and it was found in all cases that the L-form acted as a stronger inhibitor than the D-form. The inhibitions obtained with various amino acids are given in the Tables V and VI. These Tables show that α-N- or α-carboxyl-substituted amino acids as well as proline have no inhibitory action whatever. β-alanine inhibited to a markedly lesser degree than α-alanine. N-(β-DL-aspartyl)-alkylamines are powerful inhibitors of the hydrolysis of D-asparagine and D-aspartohydroxamic acid and of β-aspartyl transfer from D-asparagine to hydroxylamine. As can be seen from Table V, all the N-(β-DL-aspartyl)-alkylamines tested inhibited the hydrolysis of D-asparagine to the same extent. Table VII shows that the hydrolysis of L-asparagine and of L-β-aspartohydroxamic acid is comparatively slightly or not at all inhibited by the same amino acids that strongly inhibited the hydrolysis of the D-forms of these substrates.

The reversibility of the inhibition of hydrolysis of D-asparagine, D-aspartohydroxamic acid and of the β-D-aspartyl transfer reactions was tested in experiments with L-alanine and N-(β-L-aspartyl)-ethylamine. Figs. 6, 7 and 8 show that the inhibitions in all these cases were competitive.

K_i values for L-alanine and N-(β-L-aspartyl)-ethylamine were calculated from the data represented in the figures, using the equation: slope of the $1/V$ against $1/S$ line (in the presence of inhibitor) $= K_m/V$ max. $(1 + i/K_i)$ and the K_m value for D-asparagine obtained from Fig. 5. The K_m values for D-β-aspartohydroxamic acid and for D-asparagine in the β-aspartyl transfer reaction were established in separate experi-

TABLE VII

EFFECT OF AMINO ACIDS ON THE RATE OF HYDROLYSIS OF L-ASPARAGINE AND OF L-ASPARTOHYDROXAMIC ACID BY *Pseudomonas* EXTRACTS

Reaction mixtures, as described under *Assay procedures.* The concentration of L-asparagine and L-aspartohydroxamic acid was 0.005 *M*.

Amino acids	*Concentration*	*Hydrolysis of L-asparagine % inhibition*	*Hydrolysis of L-aspartohydroxamic acid % inhibition*
L-aspartic acid	0.005	0	20
L-aspartic acid	0.050	15	68
D-aspartic acid	0.005	0	—
D-aspartic acid	0.050	0	32
L-alanine	0.050	0	27
D-alanine	0.050	0	13
L-serine	0.050	0	26
D-serine	0.050	0	12
N-(β-L-aspartyl)-ethylamine	0.010	5	23
N-(β-D-aspartyl)-ethylamine	0.010	0	0

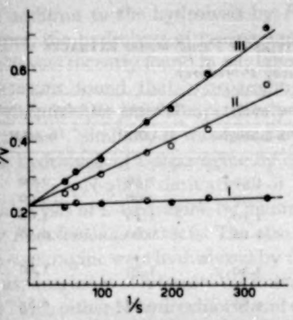

Fig. 6. Competitive inhibition of the hydrolysis of D-asparagine by L-alanine and N-(β-L-aspartyl)-ethylamine. I: D-asparagine; II: D-asparagine and N-(β-L-aspartyl)-ethylamine (0.0005 M); III: D-asparagine and L-alanine (0.006 M). S is the molar concentration of DL-asparagine. Velocity, V, is expressed in µmoles ammonia/h.

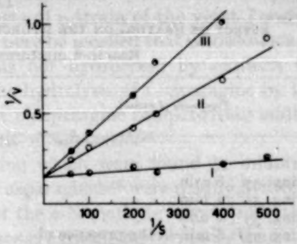

Fig. 7. Competitive inhibition of the hydrolysis of D-β-aspartohydroxamic acid by L-alanine and N-(β-L-aspartyl)-ethylamine. I: D-β-aspartohydroxamic acid; II: D-β-aspartohydroxamic acid and N-(β-L-aspartyl)-ethylamine (0.0005 M); III: D-β-aspartohydroxamic acid and L-alanine (0.008 M). S is the molar concentration of D-β-aspartohydroxamic acid. Velocity, V, is expressed in µmoles of hydroxamic acid hydrolyzed/h.

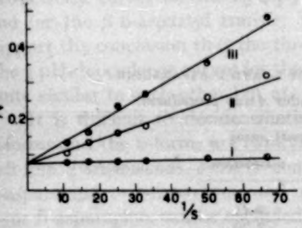

Fig. 8. Competitive inhibition of β-aspartyl transfer from D-asparagine to hydroxylamine by L-alanine and N-(β-L-aspartyl) ethylamine. I: D-asparagine; II: D-asparagine and N-(β-L-aspartyl)-ethylamine (0.0008 M); III: D-asparagine and L-alanine (0.015 M). S is the molar concentration of D-asparagine. Velocity, V, is expressed in µmoles of hydroxamic acid formed/h.

ments. The K_i values for L-alanine obtained from Figs. 6, 7 and 8 respectively were $5.0 \cdot 10^{-4}$; $7.0 \cdot 10^{-4}$ and $6.1 \cdot 10^{-4}$. For N-(β-L-aspartyl)-ethylamine the following K_i values were obtained: $6.8 \cdot 10^{-5}$; $7.9 \cdot 10^{-5}$ and $5.5 \cdot 10^{-5}$.

Heating the extracts for 8 min at 55° completely destroys the ability of the extracts to hydrolyze L- and D-asparagine, L- and D-β-aspartohydroxamic acid and the DL-α-N-ethyl derivatives of the asparagine, and to transfer the β-aspartyl group from L- and D-asparagine to hydroxylamine. On heating the extracts for 1 or 2 min at 55° the rate of hydrolysis of L-asparagine, D-asparagine, of L- or D-β-aspartohydroxamic acid decreases almost to the same extent (Table VIII). The presence during the heating (55° for 8 min) of varying amounts of one of the substrates, or of one of the compounds capable of inhibiting the hydrolysis of D-asparagine (see Table V) protects, either partially or completely, all the above-mentioned enzymic activities from inactivation. Heating at 55° for 8 min in the presence of D-asparagine or D-alanine in amounts insufficient to preserve the enzymic activities entirely caused a decrease in the rate of hydrolysis of L-asparagine, D-asparagine, L- and D-β-aspartohydroxamic acid to nearly the same extent (Table VIII).

The influence of several cations on the rate of hydrolysis of L- and D-asparagine was also investigated. It was found that Co^{++} ions, in a final concentration of 10^{-3} M, accelerate D-asparagine hydrolysis without affecting the hydrolysis of L-asparagine

TABLE VIII

EFFECT OF HEATING ON THE HYDROLYZING ACTIVITIES OF *Pseudomonas* EXTRACTS

Reaction mixtures, as under *Assay Procedures*.

Treatment of extract	*Ammonia formed µmoles*		*Hydroxamic acid hydrolyzed µmoles*	
	L-asparagine	*D-asparagine*	*L-AHA*[*]	*D-AHA*[*]
None	4.80	4.50	2.30	2.00
Heating, 55°; 8 min	0.00	0.00	0.00	0.00
Heating, 55°; 1 min	2.50	2.10	1.20	1.00
Heating, 55°; 2 min	1.35	1.10	0.60	0.50
Heating, 55°; 8 min in the presence of D-alanine (20 µmoles/ml)[**]	3.50	3.30	1.70	1.40
Heating, 55°; 8 min in the presence of D-alanine 6 µmoles/ml)[**]	1.80	1.80	0.90	0.85
Heating, 55°; 8 min in the presence of D-asparagine (5 µmoles/ml)[**]	2.95	2.80	1.40	1.25
Heating, 55°; 8 min in the presence of D-asparagine (3.5 µmoles/ml)[**]	2.25	2.05	1.10	0.95

[*] The abbreviation AHA is used for aspartohydroxamic acid.

[**] After heating in the presence of D-alanine or D-asparagine, the extracts were dialyzed in the cold against distilled water.

TABLE IX

EFFECT OF Co^{++} ON THE HYDROLYSIS OF L- AND D-ASPARAGINE

Reaction mixtures, as described under *Assay procedures*.

Concentration of Co^{++} (M)	*Ammonia formed µmoles*	
	L-asparagine	*D-asparagine*
0.000	2.60	2.45
0.001	2.65	2.70
0.005	2.55	3.45
0.01	2.60	3.75
0.05	2.50	2.60

(Table IX). This accelerating effect depends on the concentration of the Co^{++} ions. Other cations tested, namely Mn^{++}, Mg^{++}, Zn^{++}, Fe^{++} and Cu^{++}, did not influence the rate of hydrolysis of L- and D-asparagine.

From experiments conducted at different incubation temperatures it can be concluded that the hydrolysis of D-asparagine is much more temperature-dependent than the L-asparagine hydrolysis. At 38° the rates of L- and D-asparagine hydrolysis were practically the same, but at 45° D-asparagine hydrolysis velocity exceeded that of L-asparagine. At 25° L-asparagine is hydrolyzed at a velocity twice as great and at 12° 5 times as great as that of D-asparagine.

DISCUSSION

ALTENBERN AND HOUSEWRIGHT working with extracts from *Brucella abortus* observed a hydrolysis of L-asparagine as well as of D-asparagine[8]. A slight but distinct hydrolysis of D-asparagine by guinea pig serum asparaginase was described by MEISTER *et al.*[9].

In addition to the hydrolysis by *Pseudomonas* extracts as described in the present paper, the hydrolysis of D-asparagine by extracts of a strain of the yeast *Torulopsis utilis* was recently found in our laboratory[10]. It may be recalled that GROSSOWICZ AND HALPERN found that D-asparagine which was not hydrolyzed by extracts from *Mycobacterium phlei* competitively inhibited the hydrolysis of L-asparagine by these extracts[11]. Similarly it was found by KOTT that D-asparagine competitively inhibited the hydrolysis of L-asparagine by certain strains of *Salmonella*[12].

The α-N-alkyl derivatives of DL-asparagine which were found to inhibit the hydrolysis of L-asparagine by mammalian liver asparaginase[1] were readily hydrolyzed by *Pseudomonas* extracts. The observation that the α-N-methyl-, ethyl-, and ethanol DL-asparagine were hydrolyzed by the *Pseudomonas* extracts more rapidly than L- and D-asparagine is of interest. We found that in the non-enzymic hydrolysis carried out at 37° with either N-hydrochloric acid or N-sodium hydroxyde asparagine was hydrolyzed at a more rapid rate than its α-N-alkyl derivatives. α-amino acids inhibited the hydrolysis of D-asparagine and of β-D-aspartohydroxamic acid as well as the β-D-aspartyl transfer from D-asparagine to hydroxylamine. The K_i values obtained from the data given in any one of the Figs. 6, 7 and 8 for L-alanine and N-(β-L-aspartyl)-ethylamine were approximately the same. This, in addition to the similarity of the pH-activity curves for the hydrolysis of D-asparagine, D-β-aspartohydroxamic acid and for the β-D-aspartyl transfer from D-asparagine to hydroxylamine, seems to support the conclusion that the three reactions are catalyzed by the same enzyme[13]. The 3 pH-dependence curves for the 3 corresponding activities towards the L-form are quite similar to each other but are markedly different from those of the D-forms.

It is difficult to decide whether the hydrolysis and transfer reaction of the L-forms and the D-forms are catalyzed by the same enzyme. The possibility that the cell-free *Pseudomonas extracts* contain an isomerase that catalyzes the reaction L-asparagine ⇌ D-asparagine and one stereospecific asparaginase hydrolyzing either L- or D-asparagine, can be excluded. The fact that in some conditions one can obtain a much more rapid hydrolysis of D-asparagine than of L-asparagine (see Table IX) and that in other conditions the L-asparagine hydrolysis rate greatly exceeds that of D-asparagine is incompatible with this assumption. Moreover, on incubating L- or D-asparagine with *Pseudomonas* extracts containing aspartase, a maximum amount of 1 mole of NH_3 can be obtained from 1 mole D-asparagine, whereas from 1 mole of L-asparagine at least 1.5 moles of NH_3 are liberated. ALTENBERN AND HOUSEWRIGHT observed additive values of ammonia on incubating D- and L-asparagine together with enzyme preparations from *Brucella abortus*[8]. This was regarded as a strong indication that the isomers were hydrolyzed by different enzymes. In our experiments, the addition of D-asparagine to L-asparagine at saturation level did not cause an increase in the amount of ammonia formed. However, these results cannot be regarded as a proof that the hydrolysis of both asparagine isomers is catalyzed by the same enzyme, since it is possible that the hydrolysis of D-asparagine by a separate enzyme is completely inhibited by L-asparagine. The fact that heating the extracts at 55° for 1 or 2 min or heating them for a more prolonged time (8 min) in the presence of small amounts of D-alanine or D-asparagine causes a decrease in the rate of hydrolysis of L-asparagine, D-asparagine and L- and D-aspartohydroxamic acid to the same extent, suggests that all these activities are catalyzed by one and the same enzyme[13]. The difference in the pH-activity curves of L- and D-asparagine hydrolysis and the changes in the ratio between the hydrolysis velocities of the two isomers obtained under the influence of Co^{++}, or by altering the incubation temperature do not necessarily presuppose the existence of different enzymes. The points of attachment of L- and D-substrates to the same enzyme may be only in part identical and in part not. Changes in pH may affect in a different way the non-identical points of attachment, causing a difference in pH-activity curves. Similarly, the difference in effect of Co^{++} on the hydrolysis of L- and D-asparagine may be explained by difference in its way of reaction with the non-identical points of attachment.

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STUDIES ON THE MECHANISM OF FATTY ACID SYNTHESIS

VII. BIOSYNTHESIS OF FATTY ACIDS FROM MALONYL CoA

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(Received August 10th, 1959)

SUMMARY

Malonyl CoA can serve as the starting point for the synthesis of long-chain fatty acids in animal tissues. For any given tissue the rate of formation of fatty acids from malonyl CoA is many times faster than that from acetyl CoA. TPNH and one enzyme fraction (R_2) are required for the synthesis of fatty acids from malonyl CoA. DPNH is much less effective than TPNH in some tissues.

The fatty acids formed from malonyl CoA by the isolated enzyme system of cow mammary gland are similar in chain length distribution to those found in milk.

The following abbreviations are used: ATP, adenosine triphosphate; CoA, coenzyme A; DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; R_1, enzyme fraction that carboxylates acetyl CoA to malonyl CoA; R_2, enzyme fraction that catalyzes the synthesis of fatty acids from malonyl CoA.

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INTRODUCTION

In previous reports from this laboratory[1–9] the various cofactor requirements for the synthesis of long-chain fatty acids from acetate in the pigeon liver system have been described. In a more recent report[10] the requirements for the entire sequence were ultimately narrowed down to malonyl CoA, TPNH and only one fraction (R_{2gc}) of the pigeon-liver preparation. Rapid synthesis of long-chain fatty acids was demonstrated in a reaction mixture consisting of the above components.

In the present paper, data bearing on the distribution in various tissues of two enzyme fractions of the pigeon liver system for synthesis of fatty acids from acetyl CoA (R_1 and R_2*, respectively) are presented. These data suggest that malonyl CoA is widely, if not universally, used by animal tissues as substrate for the rapid synthesis of fatty acids. It is further demonstrated that the synthesis of the fatty acids present in cows' milk takes place in the mammary glands of lactating cows by way of the malonyl CoA pathway.

METHODS AND MATERIALS

Preparation of enzymes

Tissues packed in ice were obtained from freshly killed animals from the slaughter house and were used for the preparation of enzymes with a minimum loss of time.

All tissues were cleaned of extraneous materials, cut into small pieces and homogenized in a Waring blendor for 15 to 20 sec at top speed in 0.005 *M* phosphate buffer, pH 7.0 the ratio of tissue to buffer being 1 : 1.5, w/v. The resulting homogenate was centrifuged for 30 to 40 min at 6,000 × *g*. The precipitate was discarded, and the supernatant was filtered through two layers of cheese cloth. At this step most of the lipides of the mammary gland and adipose tissue preparations were easily removed since during centrifugation at 0° solid lumps of lipide are formed in the centrifuge tubes. The supernatant was then further centrifuged for 90 min at 104,000 × *g* in a Spinco Model L ultracentrifuge. The final supernatant fraction, filtered through glass wool, and the particulate fraction resuspended in the same buffer, were used as the sources of the enzymes. However, the protein concentrations of the extracts obtained from the fatty tissues (both adipose tissues and suprarenal fat) were too low for enzymic studies. The protein in these extracts was concentrated by precipitation with ammonium sulfate (60 % saturation). The precipitate was dissolved in a small volume of 0.005 *M* phosphate buffer, pH 7.0.

All manipulations were carried out between 0 to 5° with maximum speed. The enzyme preparations were tested as soon as possible and when stored were kept at —20°. Most of the activity is lost when the enzyme preparation is frozen and thawed several times. Even at —20°, 20 to 30 % of the activities were lost during storage for two to three weeks.

Assay of the enzyme activities

Determination of R_1 activity: According to the data of Tables I and II, the R_1 fraction is the limiting factor in tissues for the total synthesis of fatty acids from acetyl CoA as the starting material. The amounts of fatty acids synthesized by the tissues from acetyl CoA in the presence of all the cofactors would thus be a measure of the concentration of R_1.

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* In this paper the nomenclature for describing the two active fractions required for fatty acid synthesis is the same as the one adopted in previous communications of the series[1,4,8].

For routine assays[6,7], a mixture of 20 μmoles of potassium phosphate buffer of pH 6.5, 4.0 μmoles of $KHCO_3$, 50 mμmoles of TPNH, 0.3 μmoles of $MnCl_2$, 1.0 μmole of ATP, 30 mμmoles of [1-^{14}C]acetyl CoA (60,000 counts/min), the required amount of enzyme (usually 500 to 1000 μg of protein), and water to 0.4 ml was placed in ground-glass stoppered test tubes and incubated at 38°. The reaction was continued for exactly 10 min and then terminated by the addition of 0.2 ml of 1.0 N KOH. The reaction mixture was then heated in a water bath for 15 min, and after acidification with 0.2 ml of 2.0 N HCl, was extracted with pentane[1]. The radioactivity in the pentane extract, counted as described by WAKIL *et al.*[1], was taken as a measure of enzyme activity.

Determination of R_2 activity: The reaction mixture[10] here consisted of 20 μmoles of potassium phosphate buffer, pH 6.5, 50 mμmoles of TPNH, 6 mμmoles (12,000 counts/min) of labelled malonyl CoA, limiting amount of enzyme (usually 100 to 300 μg of protein), and water to 0.4 ml. The reaction time was at 38° exactly 10 min, and the subsequent steps were the same as those in the estimation of R_1 activity. In tissues with high R_2 activity, the amount of protein added in the assay was lowered to the point that the total radioactivity in the pentane extract did not exceed 2,000 counts/min.

WAKIL *et al.*[7] have described the method of following fatty acid synthesis spectrophotometrically by measuring the rate of oxidation of TPNH. This method is suitable for tissues containing high specific activity, but in many cases the activity was too low to be followed spectrophotometrically. The measurement of radioactivity in the pentane extracts was sufficiently sensitive to cover the entire gamut of tissue activities. For uniformity of comparison of the specific activities of all the tissues, the radioactivity in the pentane extract was used throughout.

Materials

Labelled malonyl CoA was prepared from [1-^{14}C]acetyl CoA by incubating [1-^{14}C]acetyl CoA with purified R_1 from chicken liver[10] in the presence of potassium phosphate buffer of pH 6.5, ATP, $KHCO_3$, and $MnCl_2$. Malonyl CoA formed in the reaction was purified by chromatography on paper. The preparation of [1-^{14}C]acetyl CoA and of labelled malonyl CoA will be described in more detail in another communication[11].

The sources of the following chemicals are indicated in parentheses: CoA (Pabst Laboratories); DPNH, TPNH and ATP (Sigma Chemical Company); and [1-^{14}C]-acetate (Nuclear Instrument and Chemical Corp.).

RESULTS

Distribution of R_1 and R_2 activities in various tissues

The relative distribution of R_1 and R_2 activities in the microsomal and supernatant fractions of several tissues tested, as summarized in Table I, brings out a few striking features. Compared to beef tissues, the livers of the two avian species are the most active sources of R_2. Both R_1 and R_2 can be sedimented, at least partially, by prolonged centrifugation at high speed—an indication of their particulate nature.

Finally, the specific activity of R_2 is invariably many times higher than that of R_1. This would indicate that the fatty acids are formed at a much faster rate from malonyl CoA than from acetyl CoA and that in any given tissue the former can serve as a better substrate for the rapid synthesis of fatty acids.

TABLE I

R_1 AND R_2 ACTIVITIES OF VARIOUS MAMMALIAN AND AVIAN TISSUES

Activities as mμmoles of labelled acetyl CoA or malonyl CoA incorporated into long-chain fatty acids per mg protein/10 min. Assay conditions for both R_1 and R_2 were as described in METHODS.

Tissue	*Sedimentable fractions*		*Supernatant*	
	R_1	R_2	R_1	R_2
Beef liver	—*	—	0.013	0.15
Beef brain	0.07	0.36	—	1.8
Beef pancreas	—	—	0.008	0.3
Beef lungs	0.05	0.21	0.03	0.23
Beef kidneys	—	—	0.005	0.06
Beef small intestine	—	0.015	—	0.015
Beef mammary gland	0.5	2.0	0.9	5.3
Beef adipose tissue	—	0.95	—	2.3
Beef suprarenal fat	0.24	0.90	0.65	7.0
Beef aorta	0	0	0	0
Chicken liver	0.2	1.6	0.7	14.5
Chicken ovary	—	0.02	—	0.04
Chicken oviduct	—	0	—	0
Pigeon liver	0.98	—	1.0	28.9

* Not tested.

That the formation of malonyl CoA is the limiting step in fatty acid synthesis from acetyl CoA is further demonstrated in Table II. It will be seen here that when extracts of beef liver are supplemented with R_1 from another tissue (chicken liver), the ability to form fatty acids from acetyl CoA is greatly enhanced.

TABLE II

ACCELERATION OF FATTY ACID SYNTHESIS FROM Ac* CoA BY SUPPLEMENTATION OF THE EXTRACT FROM BEEF LIVER WITH PURIFIED R_1 FROM CHICKEN LIVER[10]

The basic system contained all the components described in METHODS for assay of R_1. 3 mg of the beef liver extract were used in the assay. Total volume 0.4 ml; incubation 10 min at 38°. The R_1 of chicken liver (0.3 mg) was a purified preparation.

	Counts/min/assay
Beef liver extract (no supplement)	102
Beef liver extract (plus R_1 supplement)	734

Effect of pH on the R_2 activity of beef liver

The pH curve (Fig. 1) demonstrates sharp optimal activity at pH 6.5, and this seems to agree well with a similar curve obtained by WAKIL *et al.*[1] for the pigeon liver system with acetyl CoA as substrate.

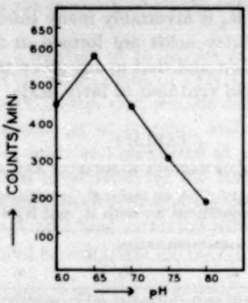

Fig. 1. Effect of pH on the R_2 activity of beef liver. Except for phosphate buffer, the concentrations of components were exactly the same as described in METHODS. Each assay tube contained 170 μg of purified beef liver extract (R_2) and 20 μmoles of potassium phosphate buffer of the pH indicated. Total volume 0.4 ml; incubation time 10 min at 38°.

Requirements for reduced pyridine nucleotides

The requirement for TPNH in the synthesis of fatty acids from malonyl CoA is demonstrated in Table III. In other experiments (not included in Table III) a similar requirement for TPNH in fatty acid synthesis from malonyl CoA was obtained with enzymes prepared from tissues like beef liver, chicken liver, etc. In the system of cow mammary gland, DPNH could replace TPNH with only partial efficiency. It may be recalled that GIBSON *et al.*[6] and WAKIL *et al.*[7] have also observed the equivalence of TPNH and DPNH in the synthesis of long-chain fatty acids from [1-^{14}C]-acetyl CoA by pigeon-liver enzymes. These results, however, differ from those of HELE, POPJÁK AND LAURYSSENS[12], who found an absolute requirement for DPNH in their system prepared from rabbit mammary gland.

TABLE III

THE SPECIFICITY OF THE PYRIDINE NUCLEOTIDE REQUIREMENT IN THE SYNTHESIS OF FATTY ACIDS FROM MALONYL CoA IN THE EXTRACT OF COW MAMMARY GLAND

The basic system contained a mixture of 20 μmoles potassium phosphate buffer pH 6.5; 6 mμmoles of malonyl CoA and extract of cow mammary gland (250 μg protein), and 50 mμmoles of TPNH or DPNH where indicated. Final volume 0.4 ml; incubation for 10 min at 38°.

Nucleotide added	*Radioactivity incorporated into fatty acids from malonyl CoA in 10 min*
None*	0
TPNH	2,015
DPNH	448
TPNH + DPNH	1,653

* The enzyme was stored at — 20° for 3 weeks.

The types of fatty acids formed from malonyl CoA by the cow mammary gland

Cows' milk contains fatty acids of varying chain lengths from C_4 to C_{18}. It was of interest, therefore, to resolve the fatty acids formed from malonyl CoA by the cow mammary gland enzyme system and to compare their relative distribution with that which has been found in cows' milk. At the end of a 10-min incubation period

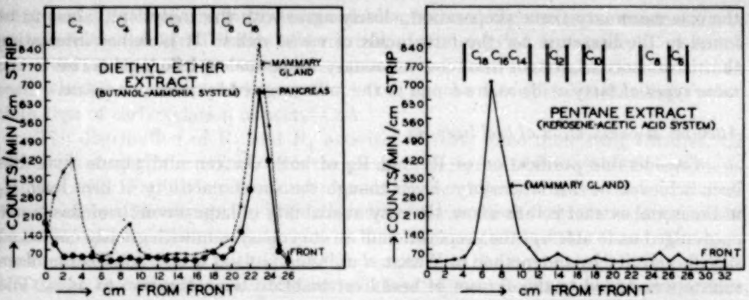

Fig. 2. Distribution of radioactivity in the fatty acids separated on the paper chromatograms of the diethyl ether (a) and pentane (b) extracts of the cow mammary gland system incubated with labelled malonyl CoA.

the fatty acids in the reaction mixture were extracted first with pentane and then with diethyl ether[3]. The short and long-chain fatty acids in the ether and pentane extracts were separated on paper according to REID AND LEDERER[13] and by reverse phase-chromatography according to KAUFMANN AND NITSCH[14].

After the chromatograms had been allowed to run for the desired length of time, the papers were dried, cut into 1-cm strips, and the radioactivity in each 1 cm strip was read in the gas-flow counter. The results, as presented in Fig. 2 (a and b), clearly demonstrate that distinct peaks for C_4 and C_6 fatty acids are obtained in the ether extracts. In this particular system of chromatographic separation (*n*-butanol saturated with 1.5 *N* ammonia), C_8 and higher fatty acids are not resolved, and they appear in one peak[3]. The same applies to Fig. 2(a). The second curve obtained from the corresponding diethyl ether extract of the fatty acids formed by another tissue like beef pancreas shows complete absence of two peaks due to C_4 and C_6 fatty acids. In Fig. 2(b) the resolution of the longer-chain fatty acids formed by the mammary gland extract, as was obtained in the pentane extract, is demonstrated. The values obtained in Fig. 2(a and b) are further analyzed in Table IV. It will be seen that the relative proportions of C_4 and C_6 fatty acids, as compared to the total fatty acids formed by

TABLE IV

THE RELATIVE CHAIN LENGTH DISTRIBUTION OF FATTY ACIDS SYNTHESIZED BY COW MAMMARY GLAND FROM MALONYL CoA

Carbon atoms in fatty acid chain	*Radioactivity*			*% radioactivity in pentane + ether extract*	*% distribution of fatty acid(s) in milk*[15]
	Pentane extract Counts/min	*Ether extract Counts/min*	*Pentane + ether extract Counts/min*		
4	—	312	312	7.0	3.5
6	33	81	114	2.6	1.7
8	57	2152	3985	90.4	94.8
10	24				
12	51				
14	1701				
16					
18					

the cow mammary tissue preparation, closely agree with the analytical values to be found in the literature for the fatty acids of cows' milk[15]. It is rather interesting that in an isolated system from cow mammary tissue malonyl CoA gives rise to the same types of fatty acids as are found in the milk secreted by the same tissue.

Attempts at purification of beef liver R_2

Considerable purification of R_1 and R_2 of both chicken and pigeon liver has been achieved in this laboratory. Even though the specific activity of beef liver R_2 in the initial extract is rather low, the easy availability of large amounts of this tissue encouraged us to attempt the preparation of an enzyme system with greatly enhanced specific activity by the method of WAKIL *et al.*[10] and GIBSON *et al.*[6]. Solid ammonium sulfate was added to the extract of beef liver to bring the saturation to 25 %. The small precipitate formed was discarded. Most of the activity precipitated between 25 and 35 % saturation of $(NH_4)_2SO_4$. This precipitate was redissolved in small amounts of 0.005 *M* potassium phosphate buffer of pH 7.0, and the solution was dialysed for 3 to 4 h against the same buffer. It was then diluted with the same buffer to a protein concentration of 30 mg/ml and treated with calcium phosphate gel[16] in the ratio of 2 mg gel to 1 mg protein. After centrifugation the supernatant was discarded and the gel was eluted 5 to 6 times with 4 volumes of 0.1 *M* potassium phosphate buffer of pH 7.0. The gel eluates were combined and again solid ammonium sulfate added to bring the saturation to 35 %. After these treatments R_1 activity was totally abolished, and a twenty-fold purification of R_2 was achieved. The enzyme (R_2), however, became very unstable at this stage, and further attempts at purification on DEAE ion-exchange cellulose columns were unsuccessful. The steps of purification are summarized in Table V.

TABLE V

PURIFICATION OF BEEF LIVER EXTRACT (R_2)

Stage in purification	*mμmoles malonyl CoA incorporated/mg protein/10 min*
After clarification of the homogenate at 104,000 × *g*	0.15
After precipitation with ammonium sulfate	1.8
After adsorption on and elution from gel	3.0

DISCUSSION

Probably one of the significant aspects of the present study is the demonstration that malonyl CoA can serve as a universal substrate for fatty acid synthesis in animal tissues. The requirements for the synthesis from malonyl CoA are much narrower than those for synthesis from acetyl CoA. Thus, whereas with acetyl CoA as the starting material, R_2, R_1, ATP, Mn^{++}, HCO_3^-, and TPNH are absolute requirements, with malonyl CoA as starting point, R_2 and TPNH would be enough to give total synthesis. Also, in any given tissue, the rate of formation of fatty acids is many times faster with malonyl CoA, as compared to acetyl CoA.

Acetyl CoA must pass through malonyl CoA before it can be incorporated into fatty acids, but once the latter compound is generated the successive condensations are relatively quicker. The rate of formation of malonyl CoA from acetyl CoA seems to be the limiting step in the synthesis from acetyl CoA. It remains, however, to be seen whether in animal bodies, malonyl CoA can be formed by mechanisms other than that of carboxylation of acetyl CoA.

The distribution of R_1 and R_2 activities reveals some interesting features. Of all tissues studied, the avian liver is the most active source of the system for synthesis of fatty acids. For obvious practical reasons, chicken liver is still the tissue of choice. The mammary gland puts out large amounts of fat in the milk which is continuously secreted, and it is not too surprising to find high activity in this tissue. The high synthesizing activity of both adipose and suprarenal fat is of considerable interest. No doubt the protein concentrations of these tissues are very low, but the fact remains that fat tissues are distributed in all parts of the body in large quantities, and they would add up to considerable amounts of synthetic activities. Attention should be drawn here to the interesting work of HAUSBERGER *et al.*[17,18] who have demonstrated that not only the adipose tissues of the rat synthesize fatty acids from labelled glucose, but the specific activity of these tissues is markedly higher than that of the liver of the same animal. The markedly higher synthetic activity of fat tissues compared to that of bovine liver with both acetyl CoA and malonyl CoA as substrates has also been borne out by the data of the present communication.

Finally, it is probably proper to note the lack of activity of the ovaries and the oviducts of laying hens, in contrast to the high activity in the cow mammary gland. This would imply that whereas at least part of the fat secreted in milk is synthesized by the mammary gland, the fatty acids in egg yolk are synthesized outside the ovaries or oviduct, most probably in the liver.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. D. E. GREEN for his encouragement and continued interest in this work. This work was supported in part by graduate training grant 2G-88 and research grant RG-5873, Division of Research Grants and research grant H-2236, National Heart Institute, NIH, USPH; National Science Foundation grant G-3227; and AEC Contract AT(11-1)-64, Project 4. Meat by-products were generously supplied by Oscar Mayer and Co., Madison. A Fulbright travel grant from the U.S. Educational Foundation in India enabled the author to accept his traineeship.

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THE IDENTIFICATION AND QUANTITATIVE ESTIMATION OF ETHANOLAMINE AND SERINE IN LIPID HYDROLYSATES

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(Received August 12th, 1959)

SUMMARY

A paper chromatographic method for the separation and identification of minute amounts (0.01–0.10 μmole) of ethanolamine and serine in lipid hydrolysates is described. The chromatograms are stained directly with a ninhydrin reagent and the resultant coloured spots cut out and extracted for quantitative estimation. The method has been applied to the analysis of several phospholipid preparations. The results are compared with those obtained by a dinitrofluorobenzene method.

INTRODUCTION

The present study was undertaken in an attempt to find a suitable method for the quantitative analysis of phosphatidylethanolamine and phosphatidylserine in phospholipid fractions. It was felt that determination of ethanolamine and serine in hydrolysates of lipids represented the most practical approach to the solution of this problem. Several such assay procedures have been described, and the field was reviewed recently by DITTMER, FEMINELLA AND HANAHAN[1], who found that many of the available methods were inadequate.

A previous paper chromatographic procedure[2] for the micro estimation of nitrogenous phosphatide constituents has been found to give good results with standard ethanolamine and serine solutions, but DITTMER[1] *et al.*[1] and MUNIER[3] have proved that it is unsatisfactory when applied to lipid hydrolysates. The chromatographic method described in this paper is both rapid and convenient, and is suitable for the determination of from 0.01 to 0.10 μmole of each compound in lipid hydrolysates.

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MATERIALS AND METHODS

Preparation of cephalin fractions from human brain

Samples corresponding to fractions III and V of human brain cephalin were prepared by the method of FOLCH[4]. Fraction III contained a large proportion of the phosphatidylserine present in the original cephalin preparation. Fraction V was rich in phosphatidylethanolamine. Both fractions, however, were mixtures of the two phosphatides and other lipid materials. The individual fractions were freed from non-lipid contaminants either by dialysis or by the washing procedure described by FOLCH, LEES AND SLOANE-STANLEY[5], and were emulsified with water, freeze-dried and stored *in vacuo* over P_2O_5.

Fraction V of human brain cephalin was further purified by chromatography on a column of silicic acid. The sub-fraction rich in ethanolamine obtained in this way was stored at —10° in chloroform solution.

Preparation of phosphatidylethanolamine fraction from ox heart

This was prepared by Dr. G. R. WEBSTER in this laboratory by fractionation of a lipid extract obtained from ox heart on a column of silicic acid.

Ethanolamine and serine standard solutions

Ethanolamine and d,l-serine were obtained from L. Light & Co. Ltd. The ethanolamine was redistilled, and the fraction boiling at 170–171° (760 mm) was used in all experiments. Stock solutions, 0.01 *M*, were made up in water and stored at 0°. Both reagents gave single ninhydrin-reactive spots on paper chromatograms.

Hydrolysis procedure

A phospholipid sample weighing from 15 to 40 mg was placed in a Pyrex test tube (15 × 125 mm) and 4 ml of 2 *N* H_2SO_4 added. The tube was sealed in a flame and then immersed in a boiling water-bath for 12 h. After cooling, the tube was opened and its contents transferred to a 12 ml Pyrex centrifuge tube. The test tube was rinsed with 1 ml water in two portions and the washes added to the original hydrolysate. 3 drops of a 0.1 % aqueous solution of methyl red were added. With frequent stirring, sufficient solid $BaCO_3$ was next added to neutralise the hydrolysate, as indicated by the cessation of CO_2 evolution and the change in indicator colour. An excess of $BaCO_3$ could be added without harmful effect. In a few samples excessive frothing occurred, but this could be prevented by the addition of a few drops of ethanol. The sample was centrifuged and the supernatant transferred to a 10 ml volumetric flask. The precipitate was washed twice with 2.5 ml portions of water, and the washes were added to the original supernatant. The volume of the hydrolysate was adjusted to 10 ml, and the samples were filtered. The clear filtrate was stored in the cold (0–4°).

With each set of phospholipid samples hydrolysed, a blank (4 ml 2 *N* H_2SO_4) and serine and ethanolamine standard solutions (20 or 40 µmoles of each in 4 ml 2 *N* H_2SO_4) were carried through all the above operations.

Paper chromatography

A 1 foot square of Whatman 3MM paper was divided into eight lanes 1.25 in. wide. 1-in. margins were left on each side of the sheet. Because of the high sensitivity of the ninhydrin reagent, it was essential that the chromatogram be handled as little as possible, and then only on the margins, particularly before colour development. The solutions to be chromatographed were applied to the paper in the centre of each lane along a base line 1 in from the bottom of the sheet. The volume applied was usually between 20 and 60 µl. The blank and serine standard solutions were applied first, as these could be dried in a blast of hot air. The volatility of ethanolamine at elevated temperatures made hot air drying impossible in the samples containing the amino alcohol.

The paper square was sewn into cylindrical form before being placed in a chromatographic chamber, and was run by the ascending technique. The solvent system employed was methyl ethyl ketone–methyl cellosolve–20% acetic acid, 40:15:20 (v/v/v). The methyl cellosolve used in this solvent was purified before use by the method of EASTOE[7] and was stored in the cold in dark bottles. The running time with this solvent was from 2 to 4 h. The papers were finally dried in air at room temperature.

Quantitative estimation of serine and ethanolamine

A ninhydrin–$SnCl_2$ reagent was made up immediately before use by a modification of the method of CLARKSON AND KENCH[8]. This was prepared by dissolving 4 mg of $SnCl_2 \cdot 2H_2O$ (Hopkin & Williams Ltd., A.R.) in 5 ml freshly-prepared 0.2 *M* citrate buffer, pH 5.0. To this was added 0.25 g ninhydrin (Hopkin & Williams Ltd.). The mixture was shaken for a few min, made up to 50 ml with isopropanol and shaken mechanically for a further 60 min.

The chromatogram was dipped in the reagent, drained free of excess liquid and placed in an oven at 50° for 90 min. After colour development the ninhydrin spots, together with appropriate areas from the blank lanes, were outlined with pencil using a template, and cut out. Thus an equal area of paper was taken for each sample. The pieces, handled with forceps, were shredded with scissors and placed in glass-stoppered test tubes. To each, 5 ml of 50% aqueous ethanol (made with redistilled ethanol) was added, and the tubes were shaken gently for 30 min. The contents of the tubes were filtered and the optical density of the filtrate was measured in 1 cm cells at 570 mµ in a spectrophotometer against 50% aqueous ethanol.

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram for which the running time was 3 h. The base line and solvent front are indicated by solid lines. Lanes A and B contained 0.06 µmole of ethanolamine and serine respectively. An aliquot of an hydrolysate of a sample of Fraction III from human brain cephalin was applied to the chromatogram in lane C. The serine spot is clearly visible, but a very faint ethanolamine spot seen on the original chromatogram is not apparent in the reproduction. The amount of ethanolamine present was less than that which could be determined accurately. In order to assay the ethanolamine content of the sample, it was necessary to apply a three-fold volume of the hydrolysate to the paper (lane D). The ethanolamine spot (circled) is now readily seen, and the quantity of ethanolamine present was sufficient for an accurate determination. Although the concentration of serine in lane D is much greater than that usually assayed, the separation of the two compounds is still distinct. An aliquot of an hydrolysate of fraction V from human brain cephalin was applied to the chromatogram in lane E. The hydrolysate contained approximately 10 times as much ethanolamine as serine. Both compounds were present in the single aliquot at concentrations suitable for analysis.

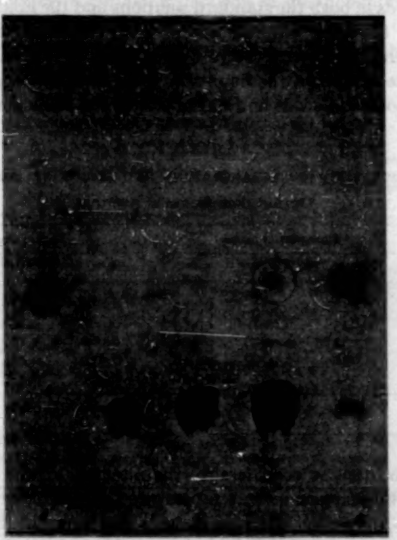

Fig. 1. A representative chromatogram. A, 0.06 μmole ethanolamine; B, 0.06 μmole serine; C and D, 20 and 60 μl respectively of an hydrolysate of fraction III from human brain cephalin; E, 20 μl of an hydrolysate of fraction V from human brain cephalin.

With the chromatographic system described the spots obtained are discrete, with little tailing. The short running time required for good separation is an advantage. The R_F values obtained for ethanolamine and serine were (mean ± S.E.M. for 26 consecutive chromatograms) 0.47 ± 0.006 and 0.22 ± 0.003 respectively.

As was reported by DITTMER *et al.*[1] for the solvent systems described by LEVINE AND CHARGAFF[2], it was found in this investigation that traces of salts or hydrochloric acid in the hydrolysates resulted in poor separation. As a consequence, the hydrolysis procedure outlined above using dilute sulphuric acid followed by neutralization with solid $BaCO_3$ was devised.

Although an appreciable loss of both ethanolamine and serine occurred during the hydrolysis procedure and the subsequent experimental manipulations, this loss was quite reproducible. It was compensated for by the fact that the standard solutions were carried through the same procedure.

In Table I the colour yields of ethanolamine and serine standards run on five separate chromatograms are recorded. The colour yield of ethanolamine on a molar basis is less than half that obtained with serine. The relationship between optical density and concentration is not perfectly linear, especially for ethanolamine. Thus it is necessary to run an appropriate set of standards for each determination. This can be done conveniently on a single chromatogram.

As can be seen from Table I, the optical densities of the standards varied considerably from one chromatogram to another. However, the colour yield of the individual compounds in both the standard solutions and the lipid hydrolysates varied correspondingly on the same chromatogram. Thus the calculated concentration of each compound in the lipid hydrolysates was considerably more consistent from one determination to the next than the figures in Table I would suggest. It is, of course, essential to apply both standards and unknowns to the same chromatogram.

TABLE I

COLOUR YIELD OF ETHANOLAMINE AND SERINE STANDARDS

(Optical density/cm at 570 mμ)

Expt. No.	*Ethanolamine (μmole)*			*Serine (μmole)*		
	0.02	*0.06*	*0.10*	*0.02*	*0.06*	*0.10*
1	0.037	0.092	0.133	0.086	0.217	0.356
2	0.035	0.082	0.119	0.068	0.193	0.319
3	0.032	0.083	0.125	0.066	0.192	0.310
4	0.031	0.069	0.118	0.070	0.192	0.317
5	0.031	0.087	0.140	0.070	0.213	0.332
Mean	0.033	0.083	0.127	0.072	0.201	0.327

It is possible to increase considerably the intensity of the ninhydrin spots on the chromatograms by increasing the temperature during colour development. With higher temperatures, however, the results were less reproducible, particularly for ethanolamine, where a marked fall in colour yield at higher concentrations was observed. Higher temperatures during colour development also resulted in very high blank readings. Using the conditions described above, blank readings for both ethanolamine and serine were of the order of 0.010 optical density unit.

In Table II the ethanolamine and serine concentrations of several phospholipid fractions are shown. The values obtained with the ninhydrin procedure described above are compared with those obtained on the same hydrolysates by means of a dinitrofluorobenzene (DNFB) method[9]. The reproducibility of the chromatographic method is indicated by the triplicate analyses on purified fraction V from human brain.

The concentrations of serine as determined by the chromatographic method were consistently lower than those obtained by the DNFB method. This is particularly apparent in the hydrolysates containing very little serine and considerable ethanolamine. We suggest that this is due to the fact that any phosphoethanolamine formed during hydrolysis would be determined as serine by the DNFB method[1]. With the chromatographic conditions used here, phosphoethanolamine has an R_F of 0.14. The colour yield of phosphoethanolamine with ninhydrin is low[10], particularly on the chromatograms. Thus this compound was not detected in the lipid hydrolysates with the ninhydrin stain. It is probably present in the hydrolysates in low and variable concentrations and could perhaps be estimated on the chromatograms by phosphorus analysis, although this was not attempted.

Choline does not react with the ninhydrin reagent. The presence of choline in the hydrolysates did not interfere with the separation of ethanolamine and serine unless it was present on the chromatograms at very high concentration (0.5 µmole or greater); it then appeared to impede the movement of the ethanolamine spot.

It is felt that a chromatographic separation of ethanolamine and serine before analysis considerably reduces the possibility of interference in their estimation by other ninhydrin-reactive substances likely to be present in lipid hydrolysates.

TABLE II

ANALYSIS OF PHOSPHOLIPID FRACTIONS

Phospholipid	*Sample No.*	*Method of analysis*	*Composition*	
			Ethanolamine %	*Serine* %
Crude fraction III (human brain)	1	DNFB	0.30	7.42
		Ninhydrin	0.47	6.94
	2	DNFB	0.29	7.94
		Ninhydrin	0.35	7.48
Purified fraction V (human brain)	1	DNFB	7.90	0.80
		Ninhydrin a	7.80	0.65
		Ninhydrin b	7.85	0.50
		Ninhydrin c	7.70	0.51
"Phosphatidylethanolamine" (ox heart)	1	DNFB	6.30	0.69
		Ninhydrin	6.34	0.25

ACKNOWLEDGEMENTS

We wish to thank Dr. G. R. WEBSTER for his gifts of the ox heart phosphatidylethanolamine fraction and of a sample of phosphoethanolamine, and for his many helpful suggestions. We wish also to thank the Medical Research Council for a grant towards the expenses of this work.

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ACCUMULATION OF TREHALOSE AND SUCROSE IN RELATION TO THE METABOLISM OF α-GLUCOSIDES IN YEASTS OF DEFINED GENOTYPE

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(Received July 21st, 1959)

SUMMARY

1. Glucose-grown haploid yeast hybrids lacking the ability to ferment sucrose and affording an extract devoid of sucrose activity failed to accumulate [^{14}C]maltose or methyl-α-glucoside but accumulated [^{14}C]sucrose up to 3 % of cell dry weight. The accumulated [^{14}C]sucrose could be removed by incubation with [^{12}C]glucose or fermentable α-glucosides. Its accumulation was inhibited by 10^{-3} *M* NaN_3 or 10^{-3} *M* 2,4-dinitrophenol.

2. Although the cells when grown on maltose or methyl-α-glucoside contained an α-glucosidase which can hydrolyse sucrose, they failed to ferment or hydrolyse sucrose. They accumulated sucrose as did glucose grown cells. The possibility that a fragile intracellular barrier separating sucrose from the α-glucosidase exists in these cells is considered.

3. The bulk of radioactivity fixed by cells incubated with ^{14}C fermentable sugars (glucose, maltose, methyl-α-glucoside) was shown to reside in trehalose. In washed cells the amount of the accumulated trehalose is fairly stable but a rapid turnover and a small accretion of trehalose occurs in the presence of the fermentable sugar.

INTRODUCTION

In a study of carbohydrases in *Saccharomyces* haploid stocks of defined genotype, Hestrin and Lindegren[2,3] found that cells grown on maltose or methyl-α-glucoside, failed to hydrolyze sucrose though the presence of sucrase activity in the cells could be demonstrated after drying or ageing. Selective permeability of cell membranes has been proposed as one of the possible explanations for this phenomenon of "cryptic" enzymic activity. Further studies of these haploids have indicated that the enzyme responsible for the hydrolysis of sucrose is the same α-glucosidase which hydrolyzes methyl-α-glucoside and/or maltose and that its formation and specificity is controlled by the yeast's genotype and the growth sugar[4]. The fact that intact cells metabolize methyl-α-glucoside and maltose but not sucrose although they contain a single enzyme

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[*] This study is part of a Ph. D. thesis submitted to the Senate of the Hebrew University, May 1958. An abstract presenting part of the results has appeared[1].

which can split these α-glucosides is consistent with the presence of a selective barrier which prevents sucrose from access to the enzyme site within the cells.

Recent work has presented evidence to the presence of specific "permease" systems which control the penetration of various substrates into cells[5]. ROBERTSON AND HALVORSON[6] have suggested the presence of an α-glucoside permease in yeasts. It seemed therefore of interest to study aspects of permeation and accumulation of α-glucosides in haploid yeasts of defined genotype in relation to the genetic constitution and to the contents and specificity of the cells inducible α-glucosidases. This communication presents results of experiments exploring these relationships.

MATERIALS AND METHODS

Yeasts: The haploid strains MA MG su mz (12836) and MA mg su mz (8293) of the Carbondale stock[3] were grown for 32 h on a rotatory shaker at 30° in 1 l batches of nutrient medium[2] containing 4 % of the desired sugar. Cells were washed three times with cold distilled water and were finally suspended in water to give 30–40 mg dry wt./ml. The cells were kept at 4° and used within 24 h except when stated otherwise.

Enzymes: Invertase concentrate was obtained from the British Drug House and diluted 1:10 in 0.1 *M* acetate buffer pH 5.0.

Levansucrase was an extract prepared from *Aerobacter levanicum*[7] cells disrupted mechanically by shaking in the Nossal disintegrator[8] for 30 sec.

Sugars: ^{14}C uniformly labelled sugars, viz. glucose, sucrose and maltose, were purchased from the Radiochemical Centre, Amersham, Bucks. Methyl-α-[^{14}C]glucoside (9.1 mC/mmole; m.p. and mixed m.p. 162°) was synthesized on a microscale from ^{14}C uniformly labelled glucose according to HELFERICH AND SCHAFER[9].

Chromatographic methods: Whatman No. 1 filter paper and the following solvent systems were employed: (a) upper layer of *n*-butanol–acetic acid–water (4:1:5, v/v); (b) *n*-butanol–ethanol–water (5:2:2 v/v). Sprays used for detection of sugar spots were: (a) *p*-anisidine–HCl for reducing sugars and β-fructosides[10]; (b) urea–phosphoric acid for ketoses[11]; (c) aqueous alkaline triphenyltetrazolium chloride for reducing sugars[12]; (d) alkaline silver nitrate for reducing and non-reducing carbohydrates[13, 14]; (e) 0.5 % ninhydrin in acetone for the detection of amino acids.

Radioautograms for detecting ^{14}C compounds on paper chromatograms were prepared by a 7-day exposure to an X-ray sensitive film. This technique enabled a margin of detection of 0.5 mμC/cm^2 of filter paper.

Quantitative methods: Total hexose content was determined by the anthrone[15] or by the phenol–sulphuric acid[16] methods. Sucrose was determined by the resorcinol method for fructose[17].

Measurements of ^{14}C accumulated in washed cells

A cell suspension (containing 9–15 mg dry wt./ml)) was incubated in 0.033 *M* K-Na phosphate buffer pH 6.8 and 0.166 *M* ^{14}C sugar (0.10–0.15 μC/ml) at 30° in a water bath with gentle agitation. Samples (1–2 ml) were withdrawn at various time intervals and the cells were collected with suction on a 1" millipore filter disc of 0.8 μ porosity (AA filter, Millipore Filter Co., Bedford, Mass.). The cake of cells was washed with two volumes of cold 0.033 *M* phosphate buffer, pH 6.8. After the addition of two drops of 15 % glycerol the disc was immediately dried under an infrared lamp, mounted on E-7A rings (Tracerlab Inc., Boston, Mass.) and measured in a conventional Geiger counter. Time needed to prepare a dry sample by this procedure did not exceed 3 min. The value of actual radioactivity was obtained after corrections, taking into account the activity of a zero time control and the value of self absorption (read from a calibration curve obtained by determining the decrease of emission in relation to the amount of dry cells on the disc).

Measurements of the water content of the yeast cells

The water space of the yeast cake was measured by a modification of known methods[18] so as to enable the use of a small amount of cells. The determination was performed as follows: A sample of a washed cell suspension (equivalent to about 20–30 mg dry wt.) in 0.033 *M* phosphate buffer pH 6.8 was collected by suction on an AA (1" diameter) millipore filter disc of known weight. The cell cake was washed with 5 ml of a 4.0 % solution of inulin and dried at 80° over P_2O_5 *in vacuo* for 10 h. The discs were weighed accurately and the dry weight of the sample and the total water content of the pressed cake (76 ± 1.5 % of its fresh weight) was obtained by difference. The dried cells were dispersed in 2 ml water and the inulin content was determined colorimetrically[17]. A sample of dried cells washed with water instead of the inulin solution served as a blank. The inulin space thus obtained was found to be 15 ± 1 % of the fresh cake weight and the weight of intracellular water calculated by difference was found to be 71.0 ± 0.8 % of the yeast fresh weight. The values presented were obtained with many samples of fresh MA MG or MA mg cells which were grown on glucose, maltose or methyl-α-glucoside.

EXPERIMENTAL

Fixation of ^{14}C by yeast cells incubated with ^{14}C sugars

The results presented in Fig. 1 show that after contact with ^{14}C fermentable sugar the cells fixed an amount of ^{14}C equivalent to 4–5 % of their dry wt. (calculated as glucose). The ^{14}C fixed could not be removed from the cells by repeated washing with cold water or a phosphate buffer of pH 6.8. Under these conditions no ^{14}C was fixed by cells unable to metabolize methyl-α-glucoside or maltose when allowed contact with these ^{14}C sugars. Only prolonged incubation (> 1 h) which might favour induction towards the utilization of these α-[^{14}C]glucosides resulted in a slow increase in ^{14}C uptake. It can be seen (Fig. 1) that the rate of net increase of ^{14}C in the cells on contact with a fermentable sugar falls gradually until it levels off to a very low value. Though MA MG cells grown on maltose fermented methyl-α-glucoside at about 1/5th the rate of maltose[3] the uptake of ^{14}C in presence of [^{14}C]maltose was about 30 % of the total amount fixed in presence of methyl-α-[^{14}C]glucoside under the same conditions of test. MA mg cells grown on maltose, *i.e.* cells which do not ferment methyl-α-glucoside nor can be induced to its utilization as a substrate, showed no uptake of radioactivity when in contact with methyl-α-[^{14}C]glucoside.

On contact with [^{14}C]sucrose an uptake of ^{14}C, which could not be removed by repeated washings, occurred. This intracellular ^{14}C uptake reaches a maximal constant value after about 1 h of incubation and was equal (when calculated as glucose) to 1.5–3.0 % of the cells' dry wt. It is interesting to note that this ^{14}C fixation in presence of sucrose occurred both in the case of glucose and α-glucoside grown cells. It will be

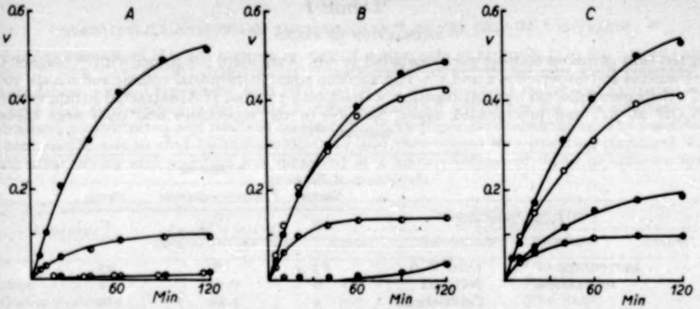

Fig. 1. Uptake of ^{14}C sugars into washed yeast cells. MA MG cells suspension was incubated under standard conditions and samples withdrawn for analysis of ^{14}C uptake as described under METHODS, v = µmoles substrate fixed/mg dry cells; ●, ○, ◓, ◐, represent [^{14}C]glucose, methyl-α-glucoside, maltose and sucrose respectively; A, glucose grown cells; B, methyl-α-glucoside grown cells; C, maltose grown cells.

recalled that in all of these cases the intact cells cannot hydrolyze sucrose though an α-glucosidase which hydrolyzes sucrose can be extracted from α-glucoside grown cells[3,4]. The total amount of ^{14}C from sucrose fixed by glucose grown cells was usually somewhat lower than the amount fixed by those grown on an α-glucoside but no constant ratio could be established.

Washed cells kept in suspension at 4° were tested at several time intervals during 14 days for their ability to fix ^{14}C when in contact with ^{14}C sugars for 1 h under the standard conditions. Only a slight decrease of ^{14}C fixation ($>$ 20 %) was found towards glucose, sucrose and methyl-α-glucoside, while with maltose the rate of ^{14}C fixation decreased at a gradual rate down to 30 % of the initial value. This phenomenon was found to accompany a decrease in maltase activity found during the ageing of the yeast suspensions (*cf.* ROBERTSON AND HALVORSON[6]). Added inorganic phosphate in the range of 0.12–0.007 *M* did not alter appreciably the amount of ^{14}C fixed by fresh washed cells.

Identification of the accumulated ^{14}C compounds

More than 80 % of the radioactivity acquired by the cells after 30 min of contact with ^{14}C fermentable sugar and all of that acquired after contact with [^{14}C]sucrose could be extracted with 5 % cold trichloroacetic acid (TCA). Most of the residual ^{14}C could be extracted with alkali at room temperature (Table I).

Cold TCA extracts were repeatedly extracted with ether, neutralized and concentrated *in vacuo*. Chromatographic analysis showed that except for traces of a very slow moving reducing component (probably a hexose phosphate) which corresponds to less than 1 % of the total sugar content when analyzed quantitatively, only a single sugar component was detected in the extracts prepared from the various cells samples. This non-reducing compound was indistinguishable from trehalose in its chromatographic behaviour. Extracts of washed cells which were in contact with sucrose showed in addition to the trehalose spot a non-reducing ketose spot which behaved chromatographically like sucrose. An amount of about 15 mg of this sugar was eluted after

TABLE I

EXTRACTION OF FIXED ^{14}C

MA MG cells grown on maltose were incubated 30 min in standard conditions with ^{14}C sugars. Cells were washed and dispersed in a cold 5 % TCA solution equal to the initial volume and shaken 30 min at 4°. Cells were collected by centrifugation, washed with 5 % cold TCA, extracted 30 min with 10 % NH_4OH at 30°, and precipitated again. Samples of the supernates and cells were taken to measure ^{14}C.

		^{14}C *substrate*		
		Glucose	*Methyl-α-glucoside*	*Sucrose*
*Amount of ^{14}C fixed**		*205*	*110*	*33*
Percentage of ^{14}C in fraction	Cold TCA	85	82	95
	NH_4OH	6	10	2
	Cell debries	5	11	0

* μmole substrate/g dry cells.

quantitative separation on sheets of filter paper. A sample treated with yeast invertase resulted in total hydrolysis to glucose and fructose as revealed by paper chromatography. A second sample was tested with levansucrase[7] and found to serve as a substrate for the formation of levan, glucose and fructose.

In addition, the chromatographic analysis of concentrates of the cold TCA extracts revealed the presence of several amino acids. Major components were identified as glutamic acid, alanine and lysine. Aspartic acid, arginine, serine and histidine were present in smaller quantities whereas about 7 other amino acids appeared in trace amounts.

Isolation of crystalline trehalose

Washed yeast cells (equivalent to about 2 g of dry wt.) were extracted for 30 min with cold 5 % TCA. After four extractions with ether the concentrated solution was applied to a 1.8 × 15 cm Darco G-60; Celite 535 column[19], washed with 500 ml water and then eluted with 500 ml 10 % ethanol. The alcoholic solution was reduced in volume in vacuo at 40°, passed through a small column of Amberlite MB-1 mixed-bed resin, and concentrated under reduced pressure into a syrup which crystallized from ethanol at 4°. Yields obtained from several yeast samples varied from 4.3 to 5.8 % of crystals with respect to cell dry weight. Recrystallization gave a material with $[\alpha]_D^{20} = +178°$ (c = 2, in H_2O); m.p. and mixed m.p. with authentic *αα*-trehalose dihydrate: 95–96°.

Location of the radioactivity in the TCA extracts

Radioautograms made from chromatograms which resolved the TCA extract of cells previously kept in contact with ^{14}C fermentable sugars for 60 min showed the radioactivity mainly at the level of the trehalose spot, in addition to a small activity in the glutamic acid and alanine spots. Quantitative studies, such as those presented in Table II show that 75–85 % of the ^{14}C found in the cold TCA extracts was recovered in trehalose and 9–15 % in amino acids. In the case of ^{14}C fixed sucrose, the only radioactive spot found in the cold TCA extracts was that of the sucrose spot, which on quantitative analysis proved to contain all of the ^{14}C fixed in the cells (Table II).

TABLE II

QUANTITATIVE ANALYSIS OF THE COMPONENTS OF THE COLD TCA EXTRACTS

An 8-ml suspension of MA MG maltose or methyl-α-glucoside grown cells (120 mg dry wt.) were incubated with 0.066 M ^{14}C sugar (about 5 mμC/μmole) at 30° for 60 min. Cells collected by centrifugation, washed repeatedly with cold 0.03 M pH 6.5 phosphate buffer and extracted with 5% cold TCA as described under Table I. After extractions with ether the TCA solutions were neutralized, concentrated and resolved quantitatively by paper chromatography. The position of ^{14}C compounds was located by radioautography and then eluted into counting planchets. This eluate after drying and counting was dissolved in a known volume of water in order to make quantitative analysis.

^{14}C substrate	(a) Per cent of fixed ^{14}C extracted with cold TCA	Isolated ^{14}C (per cent of (a))			
		Alanine	Glutamic acid	Trehalose*	Sucrose*
Glucose	74.0	4.5	3.9	87.4 (1.05)	—
Methyl-α-glucoside	73.4	6.0	10.5	77.0 (0.92)	—
Maltose	85.2	4.8	5.6	82.0 (0.66)	—
Sucrose	98.0	0	0	0 (0)	92.0 (0.94)

* Numbers in parenthesis indicate the specific radioactivity attained in the isolated material in relation to the external ^{14}C sugar employed.

Influence of various inhibitors on the ^{14}C uptake

The rate of ^{14}C uptake by washed cells in contact with either ^{14}C fermentable sugar or sucrose, could be inhibited up to 90% by $1.6 \cdot 10^{-3}$ M 2,4-dinitrophenol or sodium azide and to about 30% by $1.6 \cdot 10^{-3}$ M NaF. 0.014 M tris (hydroxymethyl) aminomethane which is a powerful competitive inhibitor of the α-glucosidase of these cells[4], did not inhibit either the fermentation or the ^{14}C uptake by intact cells. Several detergents such as cetyltrimethylammonium bromide (Cetavlon) and sodium monolauryl sulphate (Duponol) at $2 \cdot 10^{-3}$ M completely inhibited ^{14}C fixation. Sodium dodecyl sulphate and a polyoxyethylene sulphonate ether (Triton 720, Rohm and Hass Co.) at these concentrations inhibited only to a smaller extent (20–30%). Neutral surface active agents such as polyethenoxy ether (Triton x-100, Rohm and Haas Co.) and polyoxyethylene sorbitan mono-oleate (Tween 80, Atlas Power Co.) at 0.15% did not show inhibition nor a significant increase in the amount of ^{14}C fixed. It should be noted that Cetavlon at $2 \cdot 10^{-3}$ M stopped the fermentation of external sugars and microscopic examination showed incipient cell lysis[23]. In addition, treatment of ^{14}C labelled washed cells with 10^{-2} M Cetavlon resulted in the total extraction of the [^{14}C]-trehalose or sucrose[15].

Effect of ^{12}C sugars on the accumulation of ^{14}C trehalose and sucrose

From experiments such as those presented in Fig. 2 it could be seen that any dilution of the external ^{14}C fermentable sugar by the same or other ^{12}C fermentable sugars, caused a rapid decrease in the ^{14}C content of the cells. This decrease reached 80–90% of that expected from the dilution of the external ^{14}C substrate. It is evident that dilution with [^{12}C]maltose or methyl-α-glucoside usually proceeded more slowly than with glucose. [^{12}C]sucrose could dilute only the accumulated [^{14}C]sucrose but not the accumulated [^{14}C]trehalose, whereas any ^{12}C fermentable sugar caused rapid elimination of the [^{14}C]sucrose accumulated in the cells (Fig. 2). Some preliminary experiments indicated that fermentable sugar (*e.g.* glucose) caused the release of intact accumulated sucrose from the cells into the medium.

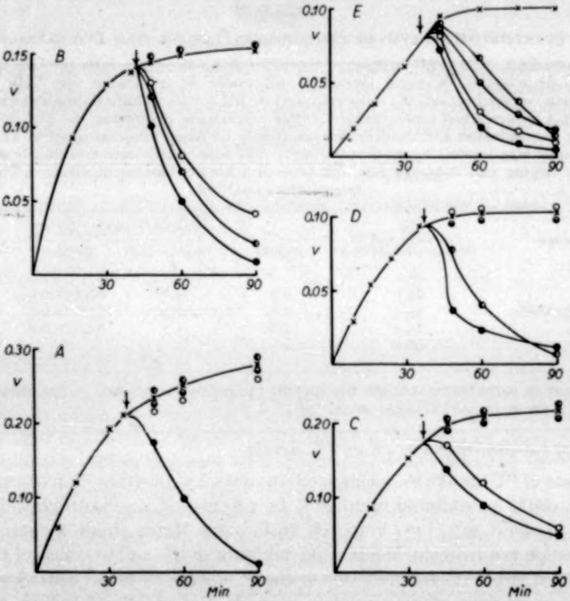

Fig. 2. Dilution of fixed ^{14}C by ^{12}C sugars. MA MG cells were incubated in standard conditions with 0.066 M ^{14}C sugars and samples withdrawn for ^{14}C analysis as described under METHODS. At time indicated by an arrow a concentrated solution of ^{12}C sugar was added to bring its concentration to 0.118 M and that of the ^{14}C sugar to 0.059 M. The values given for homologous pair of ^{12}C–^{14}C sugar are those of the experimental values corrected according to the percentage of maximal dilution expected; v = μmoles substrate fixed/mg dry wt.; x, ^{14}C uptake; ●, ○, ◓, ◑, residual ^{14}C in presence of [^{12}C]glucose, methyl-α-glucoside, maltose and sucrose respectively; A, glucose grown cells on [^{14}C]glucose; B, maltose grown cells on [^{14}C]maltose; C, methyl-α-glucoside grown cells on methyl-α-[^{14}C]glucoside; D, glucose grown cells on [^{14}C]sucrose; E, maltose grown cells on [^{14}C]sucrose.

Turnover and net synthesis of trehalose

The amount of trehalose found in fresh harvested washed cells was fairly constant (Table III) and decreased to a limited extent (< 20 %) during two weeks storage of a thick aqueous suspension at 4° either aerobically or anaerobically. It was also found that cells which accumulated [^{14}C]trehalose and were then washed free from the external sugar, lost about 20–30 % of the fixed ^{14}C within one hour but retained the rest of ^{14}C during 48 h at 30°. Likewise, the [^{14}C]sucrose accumulated in the cells showed no decrease during that period.

Incubation of the cells for periods of up to 1 h with a fermentable sugar caused an increase of 30–40 % in the net amount of trehalose (Table III). When ^{14}C sugars were used, the trehalose was rapidly labelled up to a specific radioactivity equal to that of the external ^{14}C substrate (Table II). It was found that the amount of [^{14}C]-hexose which was fixed aerobically by the resting cells during 1 h at 30° was about 15 % of the total sugar utilized (when related to the CO_2 produced under the same

TABLE III

CHANGES IN THE AMOUNT OF TREHALOSE AFTER CONTACT WITH ^{14}C SUGARS

MA MG cells were incubated 60 min under standard condition with ^{14}C sugars, washed and extracted with cold TCA as described under Tables I and II and the amount of trehalose was determined colorimetrically. Numbers indicate µmoles hexose/g dry cells.

Growth sugar	*(a) Initial trehalose in washed cells*	*Δ trehalose in relation to (a) after incubation with substrate*				*Δ sucrose*
		Glucose	*Methyl-α-glucoside*	*Maltose*	*Sucrose*	*Sucrose*
Glucose	232	+ 92	— 6	—12		+32
Methyl-α-glucoside	260	+ 82	+ 88	— 6	—11	+48
Maltose	264	+120	+116	+74		+42

conditions). The fact that at least 60 % of this ^{14}C assimilation represents turnover means that net increase of the trehalose content amounts to only about 5 % of the external sugar utilized by the resting cells.

Effect of substrate concentration on the rate of trehalose and sucrose accumulation

The uptake of ^{14}C by cells in contact with ^{14}C fermentable sugar was found to conform with the MICHAELIS-MENTEN relationship. The K_m was 0.08 M for glucose, maltose or methyl-α-glucoside as substrates (Fig. 3). This value may be taken as that of a rate limiting reaction concerned with trehalose synthesis or breakdown. It should be mentioned here that the K_m for maltose and methyl-α-glucoside as substrates of the α-glucosidase in these cells varied between 0.07–0.22 M[4], whereas K_m of fermentation in yeasts, as measured by CO_2 formation, is about ten times smaller[21, 22]. ROBERTSON AND HALVORSON[6] found a K_m of 0.12 M for fixation of cold TCA extractable ^{14}C by yeast cells in contact with methyl-α-[^{14}C]glucoside.

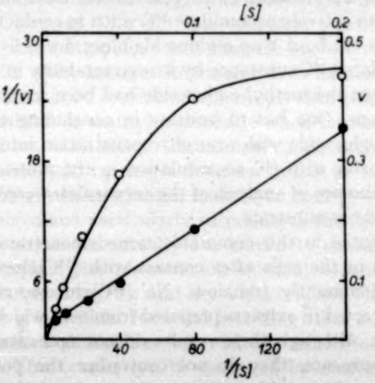

Fig. 3. Influence of sugar concentration on the ^{14}C uptake. MA MG maltose grown cells were incubated 30 min under standard conditions with varying concentration of [^{14}C]glucose and samples taken for analysis cf ^{14}C uptake as described under METHODS. S = substrate concentration; v = µmole substrate fixed/mg dry cells; ○, rate of ^{14}C uptake; ●, reciprocal values of ^{14}C uptake. Results in experiments with methyl-α-glucoside or maltose as substrates, yielded similar curves.

In contrast to the process of trehalose synthesis, accumulation of [^{14}C]sucrose was found to be a linear function of the external sucrose concentration within a wide range (0.02–0.40 *M*).

The concentration of trehalose and sucrose in the cells

Since the analysis of the water content has shown that in the washed cells there is an equivalent of 2.45 g of intracellular water per gram of dry matter, the concentration of trehalose or sucrose accumulated can be calculated assuming that they are homogeneously dissolved in the total water space of the cells. From the trehalose found in many samples of washed cells (aged 4–24 h) the values calculated were 0.050 ± 0.002 *M* for the initial and 0.075 ± 0.007 *M* for the trehalose concentration after 60 min contact with 0.166 *M* glucose or fermentable α-glucoside. While the initial concentration of sucrose in the cells was zero, it reached a value of 0.018 ± 0.02 *M* after 60 min of incubation with 0.166 *M* sucrose. It should be pointed out, however, that since these sugars are probably stored in discrete compartments within the cell and not in the whole intracellular water space, the values calculated above represent minimum values for the concentration of trehalose and sucrose accumulated by the cells.

DISCUSSION

In the light of recent evidence on the presence of specific permeases in microorganisms (*cf.* Review by COHEN AND MONOD[5]) and the suggestion that yeasts, possess a similar system controlling the entry of α-glucosides into the cells[6], the possibility could be entertained that some of the genes known to govern the utilization of glucosides (*i.e.* the MA MG or GA genes[24]) might be responsible for the permeation step and not for the formation of the α-glucosidase. LINDEGREN has opposed this hypothesis on the basis of prevailing knowledge on the utilization of α-glucosides by various genetically defined yeast hybrids[20]. ROBERTSON AND HALVORSON have shown[6] that yeast cells adapted to maltose can actively accumulate ^{14}C when in contact with methyl-α-[^{14}C]-glucoside, in amounts of about 1 μg α-glucoside/h/mg dry cells. These authors characterized the accumulated ^{14}C substance by its extractability in cold TCA and did not indicate to what extent the methyl-α-glucoside had been fermented under the same experimental conditions. One has to hesitate in concluding that maltose behaves similarly to methyl-α-glucoside with respect to penetration into the cells only on the grounds that it competes with ^{14}C accumulation in the presence of methyl-α-[^{14}C]-glucoside and in the absence of analysis of the accumulated compounds when using a fermentable α-glucoside as substrate.

The results presented in this communication demonstrate that in the haploid hybrids the ^{14}C fixed in the cells after contact with [^{14}C]glucose or a fermentable α-glucoside was predominantly trehalose. No [^{14}C]glucose, maltose or methyl-α-glucoside could be detected in extracts prepared from cells which had been in contact with these ^{14}C sugars. Although these results do not necessitate the existence of a specific α-glucoside-permease, they do not contradict the possibility that such a mechanism is operative. It is evident that in these yeast hybrids some new experimental approach has to be sought in order to detect the presence of a specific permease system for fermentable α-glucosides. Consequently, it cannot yet be decided whether the MA and MG genes are linked with the formation of a permease system.

The presence of trehalose as a carbohydrate reserve in yeast cells is well known[25,26]. The amount of trehalose and other reserve carbohydrates in washed yeast is fairly constant and their endogenous metabolism is known to be exceedingly low[26]. In addition, trehalose was shown to be one of the major components labelled by ^{14}C after baker's yeasts were brought in contact with [^{14}C]glucose[27,28]. The present results are in agreement with these observations. Trehalose was found to be the component which contained about 70 % of the ^{14}C accumulated during incubation of the cells up to 1 h with a ^{14}C-fermantable sugar. There was only a 30 % net synthesis of trehalose whereas the trehalose already existing in the cells was quickly labelled. The fast turnover of trehalose in the fermenting cells could also be demonstrated by the rapid dilution of [^{14}C]trehalose in cells when brought in contact with ^{12}C fermentable sugars. It seems probable that similarity in behaviour between the fermentable α-glucosides and glucose with respect to trehalose metabolism is due to hydrolysis by the α-glucosidase.

It is noteworthy that reaction equilibrium in the *in vitro* enzymic synthesis of trehalose phosphate from uridine-diphosphateglucose and glucose-6-phosphate has been shown to be far toward the synthesis of trehalose-phosphate and that reversibility could not be demonstrated[29]. The fact that the magnitude of the trehalose pool in the intact washed yeasts is fairly constant fits this *in vitro* result. Yet, the rapid turnover of the trehalose pool when the cells are fermenting an external hexose indicates that a highly active reaction of trehalose dissimilation is also operative. It might be probable that physiological conditions within fermenting cells favor the reversibility of trehalose synthesis by glucose transfer to uridine-diphosphate. The possibility that another enzyme which catalyses trehalose hydrolysis is operative within the fermenting cells is however not excluded. In this respect, the observations that sodium azide and 2,4-dinitrophenol cause a marked decrease in the trehalose pool of resting yeast cells[26,28,30] are of special interest.

It has been shown that washed cells, grown either on glucose or on an α-glucoside, could accumulate [^{14}C]sucrose. The fact that this accumulation could be inhibited by sodium azide and 2,4-dinitrophenol and that the sucrose once accumulated was released from the cells when brought in contact with fermentable sugars, indicate that this phenomenon cannot be considered as a simple absorption. On the other hand, cells unadapted to maltose or methyl-α-glucoside when incubated with the α-[^{14}C]-glucosides were found not to be able to accumulate these, although the possibility of their penetration into the cells by simple diffusion cannot be excluded.

The ability of cells grown on glucose, maltose or methyl-α-glucoside to accumulate sucrose is unexpected and particularly surprising in the light of the fact that the cells grown on the α-glucosides actually are known to contain a sucrose cleaving α-glucosidase[3,4]. To explain this phenomenon one may assume the presence of an intracellular barrier between sucrose and the α-glucosidase. This barrier is probably specific for sucrose as it is evident from the fact that other α-glucosides susceptible to cleavage by the same α-glucosidase (*e.g.* maltose, methyl-α-glucoside) are hydrolysed by whole cells. This barrier can be damaged by mechanical treatment such as ageing or drying of the cells thereby initiating sucrose hydrolysis. Such a change in properties of the barrier might explain some of the known instances of delayed sucrose fermentation by several strains of yeast[31].

In comparison, attention is to be drawn to some recent work concerned with the penetration of certain carbohydrates into yeast cells[32] and *E. coli*[33]. Patterns of accumulation of sugars within the cells, as was found in these studies, bear much resemblance to the phenomenon of the sucrose accumulation described in the present communication.

ACKNOWLEDGEMENT

The author is deeply indebted to Professor S. HESTRIN for much helpful advice and criticism.

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THE INITIAL PHASE OF ACTOMYOSIN-ADENOSINETRIPHOSPHATASE*

II. FACTORS INFLUENCING ACTIVITY

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(Received August 6th, 1959)

SUMMARY

1. The same initial rapid splitting of ATP by myosin B is repeatedly obtained upon adding fresh ATP, if the ATP added previously has been completely hydrolyzed.

2. The quantity of the initial rapid splitting of ITP is very small, as compared with that of ATP.

3. The initial ATP hydrolysis by non-dialysed myosin B preparations is only slightly influenced by the addition of Ca^{++} or Mg^{++}.

4. The initial burst of P-liberation is annihilated by dialysis of the myosin. It is restored by adding Mg^{++} or Mn^{++}, but not by adding Ca^{++}.

5. It is also abolished by adding small amounts of EDTA, capable of chelating only a negligible part of the Mg^{++} present. It is also decreased by the addition of *p*-chloromercuribenzoate.

6. When sufficient Mg^{++} is added to a reaction mixture containing ATP and EDTA, a burst of P-liberation is observed.

INTRODUCTION

In a previous paper[1] we described an investigation into the initial rapid liberation of P from ATP by myosin B. The phenomenon was first observed by WEBER AND HASSELBACH[2]. By means of a special apparatus constructed for the measurement of a rapid reaction, it has been revealed that the inorganic phosphate liberated in the initial rapid phase originates from the terminal phosphate group of the ATP added and that the addition of EDTA leads to complete suppression of the initial phase.

In subsequent experiments an attempt has been made to explore the reaction mechanism of the initial phase of the myosin B ATPase action more extensively, and it has been found that Mg^{++} and the SH groups of myosin B are essential for the initial burst of P-liberation.

Abbreviations: ATP, adenosine triphosphate; ATPase, adenosine triphosphatase; ITP, inosine triphosphate; P, inorganic orthophosphate; EDTA, ethylenediaminetetraacetic acid; CyDTA, 1,2-diaminocyclohexane-N,N′-tetraacetic acid; PCMB, *p*-chloromercuribenzoate.

MATERIALS AND METHODS

Myosin B was prepared from rabbit skeletal muscle according to the standard practice used in this laboratory. ATP and ITP were Sigma preparations. EDTA, PCMB and other reagents were commercial products of the best reagent grades available.

The dephosphorylation rate was measured either by using the apparatus described in detail in the previous paper[1] or by the conventional method, as occasion demanded. The intercept of the ordinate obtained by extrapolating the linear P-liberation to zero time was defined as the quantity of the initial burst. The spectrophotometric method of BOYER[3] was employed to follow the reaction of protein-SH with PCMB, assuming the molecular change of optical density at 255 mμ as 6,000.

The contents of Mg^{++} and Ca^{++} in the myosin B preparations were determined colorimetrically by a slight modification of the YANAGISAWA method[4]. The dye Plasma Colinth B was purchased from Sumitomo Chemical Co. The protein denatured in the presence of 20 % of $HClO_4$ was removed by filtration through ash-free filter paper. The filtrate was neutralized with KOH. The precipitated $KClO_4$ was also removed by filtration . The determinations were further performed exactly according to YANAGISAWA's procedure.

Other methods used were the same as described in the previous paper unless otherwise stated.

RESULTS

The relationship between the rate of the liberation of P from ATP by myosin B and the amount of the enzyme is shown in Fig. 1. It is clearly seen in this figure that the rates of P-liberation in both the steady and the initial phases are proportional to the concentration of the enzyme.

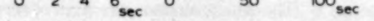

Fig. 1. Dependence of rate of liberation of P on amount of enzyme. ○, 1.8 mg; ×, 1.2 mg; △, 0.6 mg dry-weight myosin B/ml. Reaction mixture: 1 m*M* ATP, 0.6 *M* KCl, 7 m*M* Ca^{++}, pH 6.7, 23°. (a) initial phase; (b) steady phase.

A repeated rapid splitting of ATP was found upon adding fresh ATP to the reaction mixture whenever the previously added ATP was hydrolyzed completely. Similar observations have already been made by WEBER AND HASSELBACH[2]. However, the phenomenon remained to be studied quantitatively. Therefore fresh ATP was also added before the ATP added previously had completely disappeared. At various intervals after adding 50 μ*M* ATP to a series of identical reaction mixtures 1 m*M* fresh ATP was added. The result is illustrated in Fig. 2. No initial burst was observed when the remaining concentration of ATP was 25 μ*M* (arrow a), and the burst was about 25 % of the control value when the concentration of remaining ATP was 18 μM (arrow b), while the burst had a normal value again as soon as the ATP was exhausted (arrow c). From these results it is evident that during hydrolysis of ATP, the enzyme is quickly restored to its original state.

When ITP was the substrate in the presence of 10 mM Mg^{++} the initial burst was very small as compared with ATP hydrolysis in the presence of 1 mM Mg^{++} (see Fig. 3).

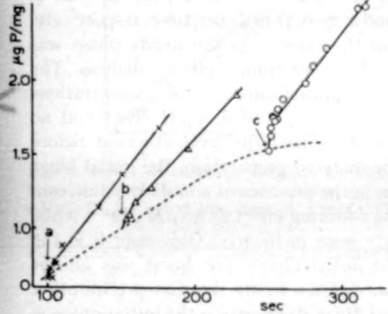

Fig. 2. Initial phase at various residues ATP concentrations. Dotted line, hydrolysis of 50 μM ATP. Arrows, additions of 1 mM ATP when concentrations of residual ATP are (a) 25 μM, (b) 18 μM and (c) 0 μM. Reaction mixture: 0.6 M KCl, 1 mM Mg^{++}, pH 6.7, 22°.

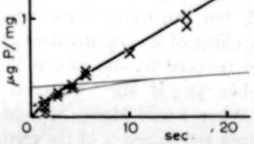

Fig. 3. Initial phase of hydrolysis of ITP. Reaction mixture: 1 mM ITP, 0.6 M KCl, 10 mM Mg^{++}, pH 6.7, 22°. Thin line, 1 mM ATP, 0.6 M KCl, 1 mM Mg^{++}, pH 6.7, 22°.

Effect of divalent cations

A typical example of the effect of the addition of Ca^{++} or Mg^{++} on ATP-hydrolysis is illustrated in Fig. 4. The activation by Ca^{++} and inhibition by Mg^{++}, which together form one of the characteristic properties of the steady phase ATPase action[5], are absent in the initial phase. The variation of the KCl concentration produced a comparable degree of effect on the initial and the steady ATPase: in the presence of

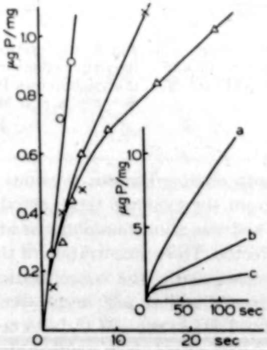

Fig. 4. Effects of Mg^{++} and Ca^{++} on initial phase. ○, 7 mM Ca^{++}; △, 4.5 mM Mg^{++}; ×, no divalent cation. 1 mM ATP, 0.6 M KCl, pH 6.7, 22°. Inserted figure, (a) 7 mM Ca^{++}; (b) no divalent cation; (c) 4.5 mM Mg^{++}.

1 mM Mg^{++} and 0.6 M KCl the velocity in the initial phase was 3 times as high as in the presence of 1 mM Mg^{++} and 0.055 M KCl, and in the steady phase 4 times.

The contents of Ca^{++} and Mg^{++} in the myosin B preparations used were 1.2–1.8 moles and 1.4–1.6 moles per 10^5 g of protein, respectively. The differences of these values from HASSELBACH's[6] (0.85 mole Ca^{++} and 0.52 mole $Mg^{++}/10^5$ g protein) will be due to the difference in the procedure of myosin B preparation. After 48 h of exhaustive dialysis of myosin B solutions against 0.6 M KCl at 0°, the amount of Ca^{++} and Mg^{++} decreased to 0.28–0.34 mole and 0.27–0.32 mole per 10^5 g, respectively. As shown in Fig. 5, no appreciable change of the velocity in the steady phase was observed, but the initial burst was abolished almost completely by dialysis. The restoring effect of several divalent cations on the initial burst, whose concentrations had been reduced by dialysis, is illustrated by Figs. 5 and 6. 5 μM Mg^{++} had no effect, while 50 μM Mg^{++} restored the burst to almost the level observed before dialysis. When 1 mM Mg^{++} was added to the dialysed preparation, the initial burst ranged from 150 to 200 % of the control value in the presence of non-dialysed myosin B. 1 mM of Mn^{++} or Sr^{++} produced the same restoring effect as 50 μM Mg^{++}, while concentrations of from 50 μM to 1 mM Ca^{++} were ineffective. Obivously it is the presence of Mg^{++} that is responsible for the initial phase. The burst was almost unaffected by three to eight repetitions of purification by the dilution–precipitation procedure. This result seems to indicate that Mg^{++} necessary to the initial phase is not removed by this procedure.

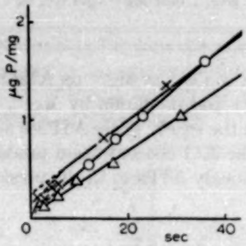

Fig. 5. Effect of dialysis on initial phase. ×, before dialysis; △, after dialysis; ○, 50 μM Ca^{++} added after dialysis. 1 mM ATP, 0.6 M KCl, pH 6.7, 21°.

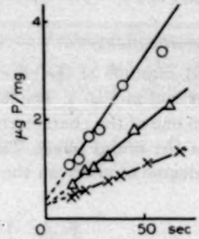

Fig. 6. Restoration of initial burst by adding divalent cations to reaction mixture containing dialysed myosin B, ×, 50 μM Mg^{++}; △, 1 mM Sr^{++}; ○, 1 mM Mn^{++}. 1 mM ATP, 0.6 M KCl, pH 6.7, 21°.

Effects of EDTA and CyDTA

The effect of EDTA already observed in our previous paper[1] was studied more thoroughly. As can be seen from the example represented in Fig. 7, the burst was reduced even at 1 μM EDTA and was almost annihilated at 10 μM EDTA, while the steady phase was almost unaffected. The concentration of the protein in these experiments was 1.4 mg/ml, and consequently the concentrations of the total and the dialysable Mg^{++} were about 20 μM and 16 μM, respectively. The inhibitory action of CyDTA was inferior to that of EDTA. 100 μM CyDTA concentration was required for the disappearance of the initial phase. It is evident from Fig. 8 that the initial phase occurs as soon as the Mg^{++} concentration is sufficiently raised in a reaction mixture containing EDTA. Furthermore, on addition of 1 mM Mg^{++} + 1 mM ATP to a reaction mixture containing 50 μM EDTA the initial burst, was identical to that observed when 1 mM ATP was added to the reaction mixture containing 1 mM Mg^{++} + 50 μM EDTA. This means that the Mg^{++}–myosin B equilibrium is reached instantaneously.

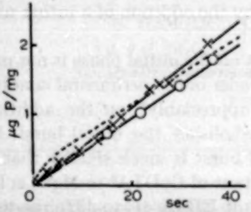

Fig. 7. Effect of chelating reagents on initial burst of P-liberation. ×, 10 μM EDTA; ○, 100 μM CyDTA; dashed line, control. 1 mM ATP, 0.6 M KCl, pH 6.7, 22°.

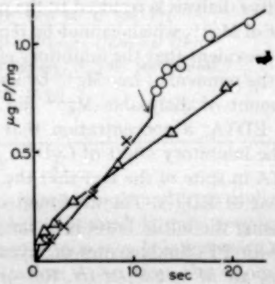

Fig. 8. Initial burst of P-liberation by adding Mg^{++} to a reaction mixture containing EDTA and ATP. ×, 50 μM EDTA and 1 mM ATP at zero time; arrow, 1 mM Mg^{++} added. △, 50 μM EDTA and 1 mM ATP, 1 mM Mg^{++} added at zero time. 0.6 M KCl, pH 6.7, 1°.

Fig. 9 shows the relationship between the quantity of PCMB bound to the SH groups of myosin B, the quantity of the burst, and the rate in the steady phase. In accordance with the results of KIELLEY AND BRADLEY[7] on myosin A, about 8 moles of PCMB were bound to 10^5 g of myosin B.

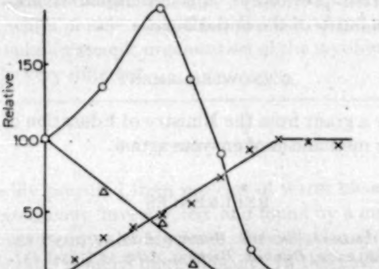

Fig. 9. Decrease of quantity of initial burst with binding of PCMB to myosin B. ×, increase of extinction at 255 mμ; ○, rate in steady phase; △, quantity of initial burst. 1 mM ATP, 0.6 M KCl, 5 mM Ca^{++}, pH 6.7, 20°.

The effect of PCMB on the steady ATPase was also quite compatible with Kielley's observations on myosin A. The most interesting point in this experiment was that a linear decrease of the burst was paraleled by a linear increase of the binding of PCMB. The burst had completely disappeared at about 80 % occupation of the SH groups by PCMB.

DISCUSSION

In the present observations the most striking feature is the effect of divalent cations on the initial phase of ATP hydrolysis: (1) using non-dialysed myosin B the rate is hardly affected by adding Mg^{++} or Ca^{++}, (2) the decrease of the burst produced by exhaustive dialysis is restored to the original level by the addition of a rather minute amount of Mg^{++}, which cannot be replaced by Ca^{++}.

It is evident that the inhibitory effect of EDTA on the initial phase is not merely due to the removal of free Mg^{++} by its chelation. Under our experimental conditions, the amount of dialysable Mg^{++} did not change appreciably* on the addition of $10\ \mu M$ EDTA, a concentration that completely abolishes the initial burst. Moreover, the inhibitory effect of CyDTA on the initial burst is much smaller than that of EDTA in spite of the fact that the binding constant of CyDTA[8] to Mg^{++} is larger than that of EDTA. The minimum concentration of EDTA (1–$10\ \mu M$) needed for suppressing the initial burst is of the same order of magnitude as the concentration of ATP-(or PP-)binding-sites of myosin B in the reaction mixture reported above, *viz.* $1.4/5 \cdot 10^5\ M = 2.8\ \mu M$ (*cf.* Ref. 9). Subsequently it seems reasonable to assume that the initial ATPase is suppressed by some complex formation between the protein and EDTA, probably mediated by Mg^{++} firmly bound to the protein.

The disappearance of the initial phase, without appreciable decrease of the rate in the steady phase, brought about by dialysis and by addition of EDTA or CyDTA is not in accordance with the presumption that the initial burst is caused by formation of an enzyme-ADP complex as an intermediate of a conventional Michaelis–Menten type, because the above supposition would unequivocally demand that the kinetic constants of the steady state must be changed whenever the initial rate is altered considerably. As reported previously[1], this conclusion is also supported by the consideration on the quantity of the initial burst.

ACKNOWLEDGEMENT

This work was aided by a grant from the Ministry of Education of Japan given to the Research Group on the mechanism of enzyme action.

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* In the presence of $1\ mM$ ATP and $16\ \mu M$ Ca^{++}, $16\ \mu M$ Mg^{++} is reduced by only a few per cent by the addition of $10\ \mu M$ EDTA.

THE ISOLATION AND PROPERTIES OF FISH MYOSIN

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(Received July 28th, 1959)

SUMMARY

A method for the preparation of myosin from carp red muscle and from mullet white and red muscles is described. The mullet myosin obtained is only very partially centrifuged off at ionic strength 0.05 and pH 7.2 at 25,000 × *g*. It is necessary to lower the pH to 6.4 in order to complete the precipitation. Apart from this anomaly, the preparations isolated from carp and mullet muscles behave as rabbit myosin from the viscosimetric, electrophoretic and ultracentrifugal points of view. The rate of sedimentation at zero concentration is 6.03 ± 0.12 S, somewhat lower than the corresponding value for cod myosin and about identical with recent values found for rabbit myosin. Electrophoresis of fish myosin in borate buffer at pH 10.5 gives much more symmetrical gradients than in phosphate buffer of pH 7.2 suggesting that the former solution could be more favourable for the study of the monomeric form of myosin. The stability of the myofibrils of white and red fish muscles in presence of extractants of high ionic strength appeared to be very different. Their disruption is easy in the case of white muscle while the behaviour of red muscle is reminiscent of rabbit muscle. The submicroscopic organisation of the myofibrils in white and red fish muscles is obviously very different.

INTRODUCTION

Myosin can be readily prepared from muscles of warm blooded vertebrates. Rabbit myosin has been extensively 'investigated' and found by a number of criteria[2–5] to be similar to rat and mouse myosin. However, the isolation of myosin from muscles of cold blooded vertebrates is much more difficult[6]. In this case, actomyosin is obtained when the methods devised for rabbit myosin are used. However, CONNELL[7] prepared cod myosin by extracting coarsely minced muscle for 5 min with a phosphate buffer of pH 6.5, containing 0.2 *M* KCl and 0.01 *M* $Na_4P_2O_7$ (total *I* 0.35). The myosin was precipitated by dialysis of the extract against water, washed with dilute phosphate buffer, redissolved and purified by reprecipitation under the same conditions. The viscometric and electrophoretic behaviour of this preparation did not differ from those of rabbit myosin, but it exhibited some polydispersity in the ultracentrifuge with a much higher and more concentration dependent rate of sedimentation. Furthermore, it had a low ATP sensitivity but this has been attributed recently to the interference of pyrophosphate[8]. One of the present authors has described another method of preparation[4,5]. Slices of frozen carp muscle, cut with a freezing microtome, were extracted for 10 min with a phosphate buffer of I 1.0 and pH 5.5. Actomyosin was removed by dialysis to I 0.25 and pH 7.2 and the myosin precipitated by dilution with water to I 0.025. From some points of view (*e.g.* solubility, viscosity, electrophoretic mobility) this preparation behaved as rabbit myosin, but on ultracentrifugation it showed two closely sedimenting peaks ($S_{20,w}$ 4.1–4.8 and 4.5–5.0 S) (*cf.* Fig. 1b). As a single peak is obtained by ultracentrifugation of carp actomyosin in the presence of ATP[5,9] some alteration of the myosin molecule or of its state of aggregation must have occurred during the preparation. In the course of the present work further results have been published by CONNELL[8]. During ultracentrifugal analysis of codling extracts of various ionic strengths, he observed a sharp peak sedimenting with the rate varying from 4 to 8 S in extracts of I 0.3 (0.05 I phosphate, 0.25 M KCl) and pH 6.5 or 7.5. This myosin was precipitated by reducing the ionic strength to approx. 0.05 and had the same electrophoretic mobility as rabbit myosin and practically zero ATP sensitivity. Its actin combining power was not very high and after precipitation two or three peaks in the range 4–8 S appeared in the ultracentrifuge. CONNELL concluded that aggregation occurs during precipitation or even in extracts held overnight at 0°. During the course of the preparation of the present manuscript CONNELL[10] has described a new preparation of cod myosin (with similar properties) by ultracentrifugal separation after dissociation of cod actomyosin with ATP or pyrophosphate. A serious disadvantage of this method is that it is not possible to obtain myosin solutions more concentrated than 0.4 %. This is because the concentration of actomyosin must not exceed 0.7 % if contamination of the myosin preparation is to be avoided. (The latter is in accordance with similar observations made independently by one of us (M.B.S.) in which myosin is separated by ultracentrifugation after dissociation of actomyosin by ATP, pyrophosphate or at high ionic strength.)

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The observation of one of us (G.H.) that myosin can also be extracted at pH 6.5 from fresh carp red muscle and the interest in this laboratory in the isolation of myosin as part of a programme on aggregation reactions has led us to reinvestigate the isolation and properties of fish myosin.

EXPERIMENTAL

Materials and methods

The earlier experimental work was carried out on specimens of a fresh water fish, mirror carp (*Cyprinus carpio* L) weighing not less than 1 kg, and was limited to red muscle of the lateral line. Later both white and red muscles from a common marine fish living in sub-tropical waters, the mullet (*Mugil cephalus* L), were separately investigated. In view of the small amount of red muscle present in both carp and mullet, four or five fish were generally used for a red muscle extract while one or two were sufficient for a white muscle extract.

The myosin preparations, obtained as described in the next section, were dialysed against a phosphate buffer of I 0.35 and pH 7.1 (0.0077 M NaH_2PO_4, 0.0308 M Na_2HPO_4, 0.25 M NaCl) and examined by several methods in this solvent.

The protein content was determined by the micro Kjeldahl method of MCKENZIE

AND WALLACE[11], assuming a total nitrogen content of 16.7 %[12]. The viscosimetric behaviour was studied in an Ostwald viscosimeter in a constant temperature bath at 1° (± 0.03°). The relative viscosity of the preparations was determined in the absence of ATP (η_{rel}) and in the presence of ATP ($\eta_{\mathrm{rel,\,ATP}}$), in order to compare their relative viscosity with that of rabbit myosin[13] and to calculate the ATP sensitivity. The latter is defined by[14],

$$\text{ATP sensitivity} = \frac{\log\eta_{\mathrm{rel}} - \log\eta_{\mathrm{rel,\,ATP}}}{\log\eta_{\mathrm{rel,\,ATP}}} \times 100$$

Ultracentrifugal measurements were carried out in a Spinco model E ultracentrifuge at 59,780 rev./min and room temperature. A Spinco phase plate was used in place of the Schlieren bar. The sedimentation coefficients were corrected to water at 20° assuming PARRISH AND MOMMAERTS'[15] value of 0.728 for the partial specific volume. Electrophoresis measurements were carried out in the cell described by DUBUISSON, DISTECHE AND DEBOT[16] and in the usual Tiselius cells. In the former, the Longsworth scanning method of recording and in the latter, the Philpot cylindrical lens method and the apparatus of MCKENZIE, ROSE AND SMITH[17], were used.

Isolation of myosin

Live fish were decapitated and skinned immediately. Both kinds of muscle were isolated carefully and brought to the cold room where they were minced without delay and the rest of the preparation carried out. Finely ground white muscle, unlike red muscle, formed a gelatinous mass when mixed with the extraction fluid and gently stirred. In order to reduce this extraction of actomyosin, coarsely minced tissue was used in the myosin extractions from white muscle. This was obtained with a 'Sunbeam' electric household mincer driven slowly. Red muscle was minced more finely, using a Baird and Tatlock (Type C18/001) tissue mincer.

The minced muscle was gently stirred for 10 min with two volumes of a phosphate buffer of pH 6.6 and I 1.0 (0.25 M KH_2PO_4, 0.25 M K_2HPO_4) or 0.5 (0.125 M KH_2PO_4, 0.125 M K_2HPO_4) containing 1 mg/ml of disodium ATP (Sigma). Comparative experiments made on mullet white muscle suggest that the increase in ionic strength of the extraction fluid from 0.5 to 1.0 does not improve the yield of the preparation and I 0.5 was used in later preparations. The role of the ATP will be discussed below.

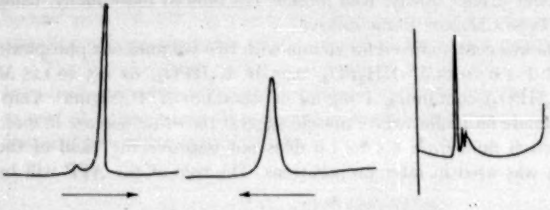
The muscle debris was separated by centrifuging at 25,000 × g for 10 min. A liquid layer of lipid material accumulated at the top of red muscle extracts and a much thinner one in the case of white muscle extracts. It was removed as completely as possible and the supernatant dialysed in Visking cellulose tubing for 18–25 h against three changes of 2.1 l of a phosphate buffer of pH 7.2 and I 0.25, obtained by dilution of the I 0.35 buffer. The use of an efficient rocking device for equilibration of the solution in order to remove completely the actomyosin present was necessary. Conductivity measurements provided a convenient check of equilibration. In the case of mullet extracts, the ionic strength of the buffer used in dialysis was lowered to 0.20 without notably lowering the yield of myosin.

Carp actomyosin was easily centrifuged off at 25,000 × g for 15 min. In the mullet preparation some slowly sedimenting material occurred even at I 0.20 and a centrifugation of 1 h at 80,000 × g in a preparative ultracentrifuge (Spinco Model L) was necessary. The clear supernatant was diluted to I 0.05 in order to precipitate the myosin. A fraction (designated fraction I) was isolated by centrifuging at $25,000 \times g$ for 10 min (carp extract) or 30 min (mullet extract). In the case of mullet extracts a second fraction (designated fraction II) was also investigated. It was precipitated by acidification of the supernatant of fraction I with dilute acetic acid to pH 6.4 (see also RESULTS). Fractions I and II were separately washed twice with 0.03 M KCl, dissolved and dialysed against the phosphate buffer of I 0.35 and pH 7.2.

RESULTS

Myosin prepared from carp red muscle

The extractions were made at I 1.0, both in the presence and absence of ATP. Fraction *I* corresponded to approx. 0.5 % of the fresh muscle weight. Its relative viscosity did not differ from that of rabbit myosin and was unchanged on addition of ATP, suggesting that it was pure myosin. However, electrophoresis and ultracentrifugation of preparations from extracts made in the absence of ATP, revealed some heterogeneity. A small amount of slow moving material could be observed on electrophoresis. Two main peaks similar to those observed in extracts at pH 5.5[4,5] and a small amount of slowly sedimenting material were observed in the ultracentrifuge. The slow electrophoretic peak was only present in very small amount in preparations from extracts made in the presence of ATP (see Fig. 1a). In the ultracentrifuge the slow sedimenting component was not shown in the latter preparations but two fast moving peaks were present in addition to the main myosin peak (Fig. 1b). The variation in the amount of these fast components from one preparation to another suggested that they correspond to various myosin aggregates.



a. b.

Fig. 1. a. Electrophoresis patterns of 0.5 % carp myosin in I 0.35 phosphate buffer pH 7.1, after 1054 min. Voltage gradient 11.84 V/cm. Ascending limb on left. b. Ultracentrifuge pattern of 0.5 % carp myosin in I 0.35 phosphate buffer pH 7.1, after 59 min at 59,780 rev./min. Phase plate angle 45°.

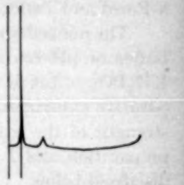


Fig. 2. Ultracentrifuge pattern of 0.87 % mullet myosin in pH 7.1 I 0.35 buffer, after 24 min at 59,780 rev./min, angle 75°, showing actomyosin (fast) peak.

Myosin prepared from white and red mullet muscle

In extracts made without addition of ATP the amount of actomyosin appeared fairly high; an appreciable precipitate forming on dialysis at I 0.25 and pH 7.2. The myosin obtained by dilution of the supernatant contained some actomyosin, having an ATP sensitivity of about 20 %. This contamination with actomyosin was also observed in the ultracentrifuge. As can be seen from Fig. 2, a small actomyosin peak has separated quickly from the much slower and very sharp myosin peak. On the other hand the myosin aggregates shown in Fig. 1b were not observed in these preparations.

Addition of ATP to the extraction fluid increased the proportion of myosin in the extracts. The actomyosin extracted could be more easily removed by dialysis at *I* 0.20 and pH 7.2. Dilution of the supernatant from *I* 0.20 and pH 7.2 to *I* 0.05 produced a notable increase in turbidity. By centrifugation at 25,000 × *g* for 30 min, the turbidity of white muscle extracts was greatly reduced but not completely removed, while water clear solutions were obtained from red muscle. The amount of myosin (fraction I) isolated under these conditions was approx. 0.3 and 0.1 % of the fresh muscle weight for white and red muscle respectively.

It was somewhat surprising that the major part of the myosin extracted did not sediment under these conditions. However, on lowering the pH of the supernatant below 6.7 further precipitation of myosin occurred. The final pH for the precipitation was varied over the range 6.1 to 6.7 and it was found that pH 6.3 to 6.4 was most suitable. Although this fraction (fraction II) was isolated at a lower pH than fraction I, it redissolved more easily in the presence of salt and gave a clearer solution (possibly due to the presence of more lipid in fraction I). Furthermore, higher yields were obtained, namely approx. 1.1 and 0.2 % for white and red muscle respectively.

Both fractions I and II from white and red muscle were examined viscometrically. The ATP sensitivities of these preparations were zero and their viscosities were similar to that of rabbit myosin.

Some ten preparations of fraction I, and fraction II were examined in the ultracentrifuge. All of these were studied in *I* 0.35 phosphate buffer of pH 7.1 at a concentration of approx. 0.9 % and some of them at several concentrations over the range 0.1–1.0 %. Phase plate angles of 40, 45 and 50° were used in order to detect more easily small amounts of any aggregated material which might be present.

In the more concentrated solution (approx. 0.9 %) all of these preparations showed a major sharp peak sedimenting at a rate characteristic of myosin. Most of them also showed a second small peak (2–3 % of the total protein) of more highly aggregated material sedimenting at a faster rate. Three of the preparations showed a small amount of a third peak of aggregated material (approx. 1 % of the total protein). The range of S values of these aggregates at a total protein concentration of 0.9 % was 4–6 S. Typical sedimentation patterns are shown in Fig. 3.

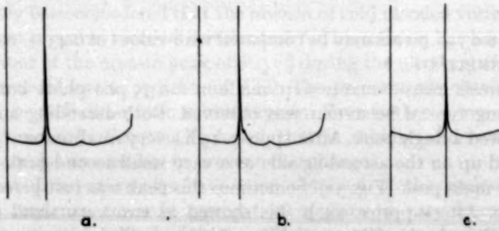


Fig. 3. Ultracentrifuge patterns of 0.9 % mullet myosin in pH 7.1, *I* 0.35 buffer. Angle 50°, speed 59,780 rev./min. a. Fraction I (preparation K_w), after 78 min. b. Fraction I (preparation L_w), after 70 min. c. Fraction II, after 110 min.

In some of the preparations at 0.5 % concentration no aggregated material was detectable, but in others it was observed. Below 0.5 % concentration no aggregated material was detectable. ATP sensitivities of all these preparations were zero.

Values of sedimentation coefficient for the myosin peak at various protein concentrations for a number of preparations are shown in Fig. 4. Fractions I and II of white and red muscle preparations were similar from the point of view of rate of sedimentation and purity. All values of S_{20} for 0.6 % and below were used to calculate the regression line:

$$S = S_0 - kC$$

where

$$S_0 = 6.03 \pm 0.12 \text{ and } k = 2.99 \pm 0.27$$

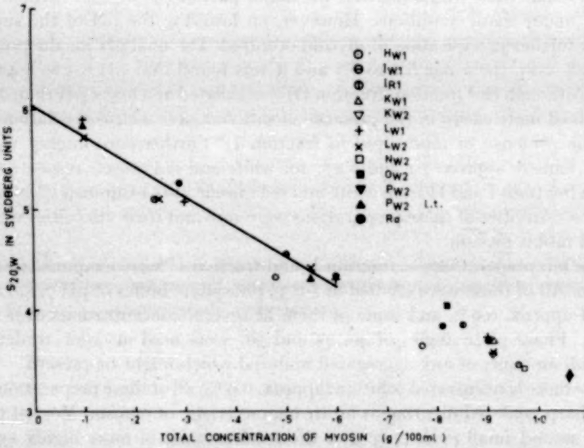


Fig. 4. Sedimentation coefficient data for various preparations of mullet myosin at different concentrations. The letters H, I etc. are laboratory identifications for different lots of fish from which the preparations were made. The subscripts R and W, 1 and 2 indicate red and white muscle preparations and fractions I and II respectively. The subscript l.t. indicates the run was done at low temperature. The three points R_a are for a preparation of rabbit myosin made in this laboratory for comparison. The regression line is drawn for values of 0.6% and below.

The S_0 value of 6.03 ± 0.12 S may be compared with values of 6.43 ± 0.08 S obtained recently by CONNELL[10].

Electrophoresis measurements were made in I 0.35 phosphate buffer of pH 7.1 and the following typical behaviour was observed. Both ascending and descending boundaries showed a single peak. After approx. 15 h a very small amount of aggregated material showed up on the ascending side as a very small second peak moving more slowly than the main peak (Fig. 5a). Sometimes this peak was just perceptible on the descending side. After approx. 24 h this showed as two very small peaks on the ascending side (Fig. 5b). It will be noted that the descending pattern is slightly asymmetrical and that the ascending peak shows an hypersharp front with an asymmetric tail. This type of pattern is observed with myosin and actomyosin[3] and is similar to that observed for associating dissociating systems (for the caseins by McKENZIE AND WAKE[20], for the lactoglobulins by OGSTON AND TILLEY[21], OGSTON AND TOMBS[22] and McKENZIE AND SMITH[23]). In TSAO borate buffer of pH 10.5 (0.104 M H_3BO_3, 0.1 M NaOH) where there should be a much reduced tendency to aggregation, the myosin pattern is much more symmetric (Fig. 5c). A single peak of normal shape was found both for the ascending and descending boundaries. This pattern is more normal than that observed by TSAO[24] for rabbit myosin at the intermediate pH of 9.0. The electrophoretic behaviour observed at pH 7.1 is not due to true heterogeneity of the preparation.

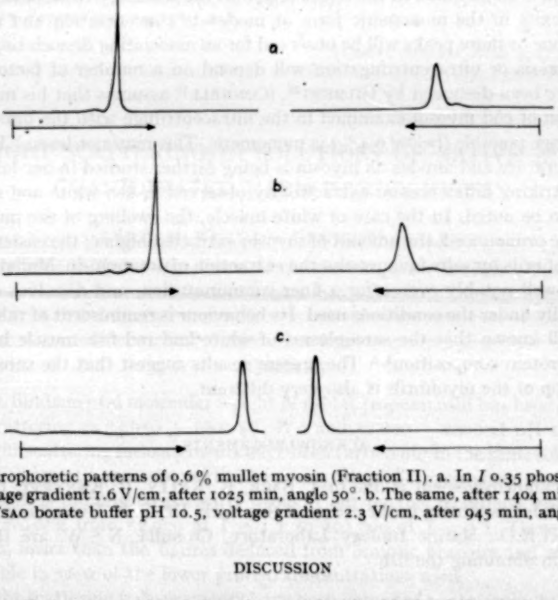


Fig. 5. Electrophoretic patterns of 0.6% mullet myosin (Fraction II). a. In I 0.35 phosphate buffer pH 7.1, voltage gradient 1.6 V/cm, after 1025 min, angle 50°. b. The same, after 1404 min, angle 40°. c. In TSAO borate buffer pH 10.5, voltage gradient 2.3 V/cm, after 945 min, angle 50°.

DISCUSSION

It has generally been considered that the myosin of cold blooded vertebrates does not differ from that of warm blooded vertebrates[3-6]. This opinion has been based mainly on the behaviour of the myosin peak observed during the ultracentrifugation of carp[9] or frog actomyosin[6] in the presence of ATP. The preparations obtained so far from frog[6], carp[4,5], or cod[7] differ from rabbit myosin. Even with cod myosin prepared by his improved methods CONNELL[10], found that it rapidly underwent side by side aggregation and he was unable to prevent this change.

Somewhat different results have been obtained in the present study of fish myosin. The tendency towards aggregation appears to vary from one species to another. Carp myosin behaves fairly similarly to cod myosin but mullet myosin appears about as stable as rabbit myosin. It can be kept for several days in the cold or be precipitated at low ionic strength without change in the ultracentrifugal pattern.

On the other hand a very partial precipitation of fish myosin at I 0.05 and pH 7.2 is observed suggesting an apparently greater solubility of this myosin. However, the two fractions isolated do not differ appreciably in viscosimetric, ultracentrifugal and electrophoretic behaviour from rabbit myosin. Preliminary results by one of us (G. H.), on fraction II indicate that prolonged centrifugation at $100,000 \times g$ will bring down most of this "soluble" fraction at pH 7.1. In any case, fish myosin appears to form at low ionic strength and neutral pH an interesting colloidal system worthy of further investigation. The difference in electrophoretic patterns at pH 7.1 and 10.5 is also of interest. It seems unlikely that this is due to irreversible changes. Electrotitration[26,27] has shown that rabbit and cat myosin are stable in this pH range. A much more likely explanation is to be found in the effect of pH on the tendency to association. Myosin may not exist in the monomeric form at moderate concentration and neutral pH. Whether one or more peaks will be observed for an associating dissociating system on electrophoresis or ultracentrifugation will depend on a number of factors, some of which have been discussed by GILBERT[25]. (CONNELL[10] assumes that his monodisperse preparation of cod myosin examined in the ultracentrifuge with the limited concentration range possible (below 0.4%) is monomeric. This may not be so.) The effect of pH, temperature and amides on myosin is being further studied in our laboratory.

The striking differences of extractibility observed in fish white and red muscles should also be noted. In the case of white muscle, the swelling of the muscle brei is much more pronounced, the amount of myosin extracted higher; the easier disruption of the myofibrils by salts favours also the extraction of actomyosin. Mullet red muscle does not swell notably even after a finer comminutation, and dissolves only slowly and partially under the conditions used. Its behaviour is reminiscent of rabbit muscle. It was well known that the sarcoplasms of white and red fish muscle have a very different protein composition[4,5]. The present results suggest that the submicroscopic organization of the myofibrils is also very different.

ACKNOWLEDGEMENTS

Grateful acknowledgement is due to J. BACK, K. KELLY, B. KENNETT, L. ROBERTS and S. ROSE for assistance in the course of this work. Dr. G. F. HUMPHREY and officers of the C.S.I.R.O., Marine Biology Laboratory, Cronulla, N.S.W. are thanked for assistance in obtaining the fish.

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LIGHT SCATTERING IN SOLUTIONS OF NATIVE AND GUANIDINATED RABBIT TROPOMYOSIN

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(Received August 10th, 1959)

SUMMARY

1. The fundamental molecular weight of rabbit tropomyosin has been established by light scattering as 54,050 ± 1000 at pH 2 and 52,600 ± 1000 at pH 12.

2. Light scattering measurements have been carried out in the same solvent media as the earlier osmotic pressure work. The aggregation of tropomyosin particles as the ionic strength is diminished is clearly brought out by this technique, the molecular weight increasing from 52,900 at $I = 1.1$ to 101,000 at $I = 0.1$. These values are about 20 % lower than the figures deduced from osmotic pressure and are probably more reliable in view of the lower protein concentrations used.

3. Light scattering measurements have been extended to the ionic strength range below 0.1 where aggregation is most pronounced. The data suggest that up to the hexamer stage the polymerization process is end-to-end.

4. The preparation and characterization of a guanidinated derivative of rabbit tropomyosin by reaction with S-methylisothiourea are described.

INTRODUCTION

Previous investigations have shown that the particle weight of rabbit tropomyosin, owing to its reversible polymerization and depolymerization, is a sensitive function of the ionic strength of the dispersing buffer. BAILEY, GUTFREUND AND OGSTON[1] estimated the molecular weight by sedimentation-diffusion and osmotic pressure measurements as 90,000 for a solution of pH 6.5 and ionic strength 0.27. TSAO, BAILEY AND ADAIR[2], using the osmotic pressure technique, found that only in strongly depolymerizing media such as 6.7 *M* urea and 0.01 *N* HCl, could the monomeric molecular weight of 53,000 be obtained. Measurements in salt solutions of ionic strengths from 0.1 to 1.1 at pH 6.5 showed that the average molecular weight varied from 135,000 to 65,000, diminishing with increasing salt concentration. DOTY AND SANDERS[3], in a light scattering study, duplicated the experimental conditions of TSAO *et al.* and found that at ionic strength 0.1 the particle weight was 100,000, decreasing to 55,000 at ionic strength 1.1. These values are about 20 % lower than the corresponding values derived from osmotic pressure and the difference is well outside the range of experimental error of the two methods. In order to clarify this apparent discrepancy it was felt desirable to repeat the light scattering work and to extend the measurements to the ionic strength range below 0.1 where aggregation is most pronounced. In this region of higher molecular weights, light scattering is vastly superior to osmotic pressure, since the intensity of scattering goes up with increasing molecular weight whereas osmotic pressure goes down.

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The second aspect of this study is concerned with the preparation and properties of guanidinated rabbit tropomyosin. One of the major analytical differences between rabbit tropomyosin and *Pinna nobilis* tropomyosin is the replacement of the lysine residues of the former by arginine in the latter. It has been suggested that this amino acid difference may be a reflection of physico-chemical differences between the two proteins[4, 5], since rabbit tropomyosin is of the water-soluble variety and tends to aggregate in the absence of salt ions while *Pinna* is of the less soluble globulin type and shows no polymerizing tendency. In order to test this suggestion the lysyl residues of rabbit tropomyosin were converted to homoarginine ones by reaction with S-methylisothiourea. Some of the physico-chemical properties of the resulting guanidinated derivative are reported in this paper.

METHODS AND MATERIALS

Preparation of native protein samples

The isolation of rabbit tropomyosin followed the procedure of BAILEY[6], with isoelectric precipitation of the protein from the muscle extract at pH 4.6 to 4.8, and subsequent salting out in the 41 to 70 % $(NH_4)_2SO_4$ saturation range. Purification was achieved by reprecipitation of the protein and resalting out with $(NH_4)_2SO_4$. It was then stored at 0° as an $(NH_4)_2SO_4$ paste. Prior to use, the paste was dissolved in and dialyzed against water until free of $(NH_4)_2SO_4$, and then equilibrated against several changes of the outer liquid to be used in light scattering measurements. Protein concentrations were determined by either the micro-Kjeldahl method, taking 16.7 % as the nitrogen content of the protein[6] or interferometrically, using a value of 0.188 for its refractive increment (see below).

Guanidination of rabbit tropomyosin

A modified form of the procedure of ROCHE, MORGUE AND BARET[7] was used. A three-times crystallized preparation was dissolved and precipitated at pH 5 with

0.1 *M* acetate buffer, brought to pH 9 with *N* NaOH and cooled in ice. 10 ml (approx. 0.17 g protein) were then mixed with 0.7 g S-methylisothiourea (cyanamid). 1 ml of concentrated NH_3 was now added to bring the pH to 10. A control without addition of the urea derivative was treated similarly and both were left for 2 days at 4°. Each was then dialyzed against several changes of a solution containing 16 g $(NH_4)_2SO_4$ in 0.01 *M* acetate buffer pH 5.4. The control sample precipitated as a mixture of amorphous and crystalline material showing that the alkali treatment in itself had caused no extensive denaturation, while the treated portion was entirely amorphous.

Estimation of guanidino groups

The SAKAGUCHI method[8] as modified by CHERVENKA AND WILCOX[9] was applied after hydrolysis of the protein for 6 h with 6*N* HCl at 100°. Both with edestin and with untreated rabbit tropomyosin the method gave values rather higher than those obtained by the STEIN-MOORE method, *e.g.*, the SAKAGUCHI method gave values of 17.6 and 9.2 g arginine/100 g protein for edestin and tropomyosin as compared with the STEIN-MOORE values of 16.8 and 7.8 respectively. The number of lysine groups remaining unsubstituted could, however, be determined directly by preparing the DNP-protein, hydrolyzing and estimating the DNP-lysine after extraction into ether as the ε-DNP-α-methoxycarbonyl derivative[4].

Extent of guanidination

The percentage of guanidino groups calculated as arginine after correction for overestimation by the SAKAGUCHI method was 25.4, corresponding to 146 residues/10^5 g protein. In fully guanidinated tropomyosin, allowing for the increase of molecular weight by addition of amidine groups, there should be 105 homoarginine and 40 arginine residues. The SAKAGUCHI method thus indicates that guanidination was complete, but the direct estimation of lysine by the FDNB method revealed a lysine content of 1.9 % corresponding to 13 residues/10^5 g protein. Taken together, however, the two methods do suggest a high degree of substitution, *i.e.*, at least 132 out of a possible total of 145 residues/10^5 g protein contain guanidino groups.

Optical measurements

Some of the light scattering measurements at 90° at a wavelength of 546 mμ were carried out in an apparatus described by GORING AND JOHNSON[10]; others were performed with a Brice-Phoenix photometer, using an opal glass primary standard for the determination of absolute turbidities[11]. Solutions of tropomyosin which had been previously clarified by initial high speed centrifugation (20,000 × *g*) for 30 min were filtered either through collodion membranes or millipore filters of pore size 0.45 μ prior to the measurements. Similarly determined turbidities on the pure solvents were subtracted from the readings obtained with tropomyosin solutions. Scattering dissymmetries at either 60° and 120° or 45° and 135° were determined concurrently with the 90° intensities. Depolarization was found to be negligible; hence no depolarization correction was applied.

The refractive index increment, d*n*/d*c*, of both native and guanidinated rabbit tropomyosin was determined in a Zeiss interferometer using white light ($\lambda = 5600$ Å) to label the zero-order band. These measurements were corrected to 5461 Å using the dispersion equation of PERLMANN AND LONGSWORTH[12]. The value of d*n*/d*c* for both proteins was indistinguishable being 0.188 at $\lambda = 5461$ Å and 25°; this yielded a value of $3.95^{\circ}\ 10^{-6}$ for the Debye factor, H, in the light scattering equation.

Weight-average molecular weights were calculated by use of the well-known equation for small symmetrical scatterers[13]:

$$\frac{Hc}{\tau} = \frac{1}{M} + 2\,Bc \qquad (1)$$

A modified form of this equation was used in the case of those solutions exhibiting appreciable angular dissymmetries of scattering due to the phenomenon of intraparticle interference[14] viz:

$$\frac{Hc}{\tau_{\text{corr.}}} = \frac{1}{M} + 2\,Bc. \qquad (2)$$

where $\tau_{\text{corr.}} = \tau \cdot P(90)^{-1}$ and P(90) is a particle scattering factor accounting for the decrease of scattering due to intraparticle interference and derivable from the dissymmetry ratio Z. Dissymmetry ratios were also used to evaluate molecular lengths in accordance with the graphic solutions given by DOTY AND STEINER[15] and JOHNSON[16].

RESULTS AND DISCUSSION

Molecular weight of the fundamental monomer

In order to establish the monomeric molecular weight by light scattering, experiments were carried out in two media known to favor depolymerization: acid at pH 2 and alkali at pH 12. The data, plotted in the conventional manner as the dependence of the turbidity function Hc/τ on c, are shown in Fig. 1. The observed dissymmetries of scattering were 1.04 and 1.05 in acid and alkali respectively. Since these values are well within experimental error no correction was applied to the observed turbidities. The extrapolated values of Hc/τ, $1.85 \cdot 10^{-5}$ at pH 2 and $1.92 \cdot 10^{-5}$ at pH 12, correspond to molecular weights of 54,050 ± 1000 and 52,600 ± 1000 respectively. These values are in good agreement with the molecular weight of 53,000 found by osmotic pressure for rabbit tropomyosin in 0.01 N HCl and in 6.7 M urea[2].

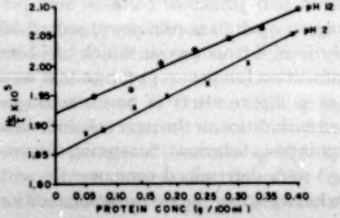

Fig. 1. A plot of Hc/τ as a function of concentration for rabbit tropomyosin: ×, in acid at pH 2; ⊙, in alkali at pH 12.

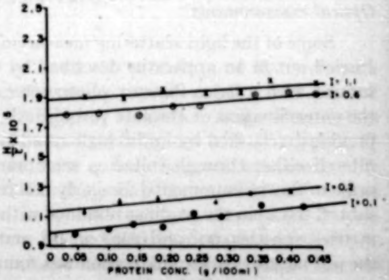

Fig. 2. Light scattering of rabbit tropomyosin in NaCl–phosphate buffer solutions. Solvents: (a) ●, 0.06 M phosphate buffer, pH 6.5, I = 0.1; (b) ▲, 0.06 M phosphate–0.1 M NaCl, pH 6.5, I = 0.2; (c) ⊙, 0.06 M phosphate–0.5 M NaCl, pH 6.5, I = 0.6; (d) ×, 0.06 M phosphate–1.0 M NaCl, pH 6.5, I = 1.1.

Aggregation of rabbit tropomyosin

The first group of experiments were carried out in the same solvent media as used in the osmotic pressure work. Accordingly, the protein was dialyzed against NaCl solutions of varying concentration, each containing 0.06 *M* phosphate buffer, pH 6.5. Representative experiments at ionic strengths 0.1, 0.2, 0.6 and 1.1 are shown in Fig. 2 and a summary of all the results is given in Table I.

Since the observed dissymmetries of scattering did not differ significantly from unity, the fundamental DEBYE equation was used directly in the calculation of molecular weights. The aggregation of tropomyosin particles as the ionic strength is diminished is clearly brought out by this technique, the molecular weight increasing from 52,900 to 100,500 over the ionic strength range covered. It is also to be noted that these values, which substantiate those reported by DOTY AND SANDERS, are about 20 % lower than the values obtained by osmotic pressure under comparable conditions. However, greater reliability should be placed on the light scattering measurements in view of their accessibility to a protein concentration range 10 times smaller than that attainable with osmotic pressure (0.05 % as compared with 0.5 %); hence with light scattering less uncertainty is associated with the extrapolated ordinate intercepts.

TABLE I

AGGREGATION OF RABBIT TROPOMYOSIN OVER THE IONIC STRENGTH RANGE 0.1 TO 1.1

Solvent system	*pH*	*Ionic strength*	*Scattering dissymmetry Z*	$\left(\frac{Hc}{\tau}\right)_{c \to 0}$	$M_{\text{light scattering}}$ (*This study*)	$M_{\text{o.p.}}$ (TSAO *et al.*)	$M_{\text{light scattering}}$ (DOTY AND SANDERS)
0.06 *M* phosphate	6.5	0.1	1.06	0.99	101,000	135,000	100,000
0.06 *M* phosphate–0.1 *M* NaCl	6.5	0.2	1.03	1.13	88,500	111,000	
0.06 *M* phosphate–0.5 *M* NaCl	6.5	0.6	1.05	1.78	56,200	67,000	
0.06 *M* phosphate–1.0 *M* NaCl	6.5	1.1	1.01	1.89	52,900	64,500	55,000

In order further to explore the aggregation phenomenon, scattering-concentration dependencies were determined for rabbit tropomyosin at ionic strengths 0.04, 0.02 and 0.01. All three solutions exhibited appreciable dissymmetry of scattering as is shown in Fig. 3. Since the dissymmetries showed no perceptible dependence on protein concentration they were averaged at the different concentrations. These average values were then used to calculate molecular lengths for tropomyosin, assuming different possible particle shapes, by means of the graphs given by DOTY AND STEINER[15]; the results are collected in Table II.

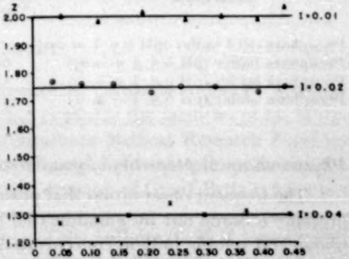

Fig. 3. A plot of the dissymmetry function *Z versus* protein concentration for rabbit tropomyosin in phosphate buffer of pH 6.5 and ionic strengths 0.01 (▲), 0.02 (⊙) and 0.04 (×).

TABLE II

MOLECULAR LENGTH OF RABBIT TROPOMYOSIN FOR DIFFERENT POSSIBLE PARTICLE SHAPES

Solvent system	$Z = \frac{I_{45^\circ}}{I_{135^\circ}}$	Rod-shaped model (Length, Å)	Random monodispersed coil (Length, Å)	Polydispersed coil*
Phosphate buffer (pH 6.5, I = 0.04)	1.30	1305	898	877
Phosphate buffer (pH 6.5, I = 0.02)	1.75	2040	1408	1306
Phosphate buffer (pH 6.5, I = 0.01)	2.00	2448	1632	1510

* Root mean square of distance between ends of coil, Å.

Of the 3 possible particle shapes, the rod-model is the most probable for rabbit tropomyosin since recent optical rotatory dispersion measurements suggest that the greater part of its molecule is folded in the α-helical form[17]. Therefore in all further discussions, we shall consider the rabbit tropomyosin molecule as a rod-shaped entity.

Because of the appreciable angular dissymmetry of scattering shown by the tropomyosin solutions, it was necessary to multiply the turbidity values, as obtained by measuring the scattered light at 90°, by the reciprocal of the particle scattering factor, $P(90)^{-1}$. From the DOTY-STEINER graphs the factors for correcting the turbidity for rods of the above specified lengths are 1.27, 1.68 and 1.88 (corresponding to ionic strengths 0.04, 0.02 and 0.01 respectively). The corrected turbidity values were then used in equation (2) to evaluate weight-average molecular weights. The values of $Hc/\tau_{corr.}$ are plotted as a function of c in Fig. 4, where the best straight lines have been drawn by the method of least squares. The limiting ordinate intercepts and their corresponding molecular weights are listed in Table III.

Taking the molecular weight of the monomer as 53,000 and its length as 400 Å[2], the above data suggest that the aggregation process has extended to the hexamer level over the ionic strength range 1.1 to 0.01. Furthermore the aggregation appears to be end-to-end at least to the hexamer stage in view of the essential constancy of the ratio molecular weight/length (M/l). If the aggregation were side-by-side the ratio of M/l would have increased six-fold over this ionic strength range. Thus these data reaffirm an earlier conclusion[18] that the polymerization appears to be electrostatic in origin and essentially linear, at least in the early stages.

TABLE III

AGGREGATION OF RABBIT TROPOMYOSIN OVER THE IONIC STRENGTH RANGE 0.04 TO 0.01

Solvent system	$\frac{Hc}{\tau_{corr.}} \cdot 10^6$	M	Length, l (Å)	$\frac{M}{l}$
Phosphate–KCl buffer (pH 6.5, I = 1.1)		52,900	400	132
Phosphate buffer (pH 6.5, I = 0.04)	6.48	154,300	1305	118
Phosphate buffer (pH 6.5, I = 0.02)	3.83	261,100	2040	128
Phosphate buffer (pH 6.5, I = 0.01)	3.18	317,600	2448	129

Physico-chemical properties of guanidinated rabbit tropomyosin

The frequent observation that protein derivatives are less soluble than the parent protein[19] is borne out by guanidinated tropomyosin. The treated material was amorphous and soluble only in concentrated urea solution or in acid at pH 2; it was quite insoluble in neutral solution even with added salt. Of interest in this connection are

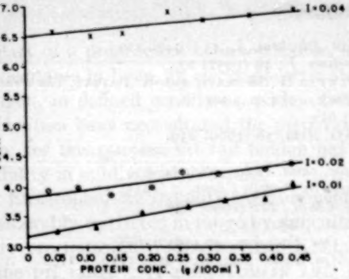

Fig. 4. $Hc/\tau_{corr.}$ *versus* c data for rabbit tropomyosin in phosphate buffer of pH 6.5 and ionic strengths 0.01 (▲), 0.02 (⊙) and 0.04 (×).

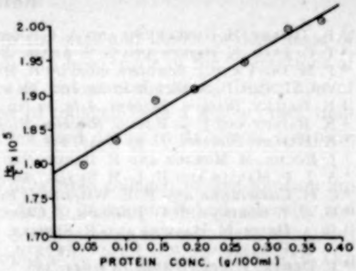

Fig. 5. A plot of Hc/τ *versus* c for guanidinated rabbit tropomyosin in acid at pH 2.

the findings of TAN AND TSAO[20] who, after treating rabbit tropomyosin with O-methylisourea, studied the effect of varying degrees of guanidination on solubility and polymerizability. The protein remained reasonably soluble in salt solutions when 20–40 % of the ε-amino groups were substituted, but with further substitution a marked decrease of solubility and salting out occurred between ionic strength 0.02 and 0.04.

Sedimentation measurements were carried out in a Spinco model E analytical ultracentrifuge. A single symmetrical peak indicated that the derivative was homogeneous at pH 2 and ionic strength of 0.10. The calculated $s_{20,w}$ of 3 S agrees very well with that of native rabbit tropomyosin.

The results of a scattering-concentration dependency study on the guanidinated protein in acid at pH 2 are summarized in Fig. 5. A molecular weight of 55,900 ± 1000 was calculated from the limiting ordinate intercept of $1.79 \cdot 10^{-5}$. This molecular weight is greater by about 1850 than that obtained for the native protein in the same solvent system (see Fig. 1) and corresponds to the addition of some 44 amidine groups. Although little quantitative significance should be attached to this figure in view of the smallness of the change relative to the experimental error associated with the method, the observed variation is in the right direction. It is also to be noted that the slope of the above plot, $d/dc\ (Hc/\tau)$, which is proportional to the interaction constant B, has a value of $6 \cdot 10^{-3}$ as compared to $5 \cdot 10^{-3}$ for the native protein in the same medium. This virtual identity of slope in the 2 cases suggests that the guanidination process has produced no drastic change in the charge pattern of the protein.

ACKNOWLEDGEMENTS

One of the authors (C. M. K.) would like to express his gratitude to Dr. P. JOHNSON of the Department of Colloid Science for making available the facilities of his laboratory and for helpful advice and to the Life Insurance Medical Research Fund for a grant which enabled the purchase of a Brice-Phoenix light scattering photometer. Thanks are also due to the American Cyanamid Company of Great Britain for a gift of S-methylisothiourea.

This investigation was carried out while one of the authors (C.M.K.) was holder of a Life Insurance Medical Research Fund Postdoctoral Fellowship, 1956–1957.

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Biochim. Biophys. Acta, 40 (1960) 149–156

MICROELECTROPHORETIC STUDIES OF SOLUBLE COLLAGEN

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(Received August 12th, 1959)

SUMMARY

The electrophoretic mobility of soluble collagen of rat tail tendon, adsorbed on oil droplets, has been measured in a range of salt solutions between pH 1 and pH 12, maintained at constant ionic strength. Specific effects of the lower valent buffer ions used appear small and the corresponding mobilities lie on a smooth curve. These mobilities parallel the hydrogen ion binding expected from the amino acid composition, with a long isoelectric zone in the range pH 7.7 ± 0.8, in agreement with reported isoelectric points of native collagen.

Addition of the divalent cations: Pb^{++}, Cu^{++}, Ca^{++}, Mg^{++}, or of the anions: citrate, phosphate, pyrophosphate, oxalate, at constant ionic strength shifts the mobility, in order of decreasing effect, to more positive or negative values respectively, with consequent shortening and raising or lowering of the isoelectric zone. These specific effects are attributed to binding of these ions by the protein, and the quantitative interpretation is discussed.

INTRODUCTION

As part of a programme of investigation into the sites and modes of deposition of radioisotopes in bone, an electrophoretic study has been made of the interactions of collagen, in defined conditions, with a range of anions and cations. Difficulties of purification have necessitated the preparation of collagen from a source other than bone; for this purpose rat tail tendon has been used owing to its almost complete solubility in mildly acid solutions.

Electrophoretic mobility measurements of soluble collagen in free solution are considerably restricted in range by difficulties due to its extremely high viscosity and ready precipitation[1-3]. BROWN AND KELLY[1] confined their mobility determinations to the pH range 3.55–4.86; TOMLIN AND TURNER[2] to pH 3.2; and BENSUSAN AND HOYT[3] to a single pH of 8.3 in a stabilising buffer solution. On the other hand microscopic electrophoresis, in which soluble material is adsorbed on small particles, allows rapid measurements in extremely dilute solutions, with consequent minimisation of effects of viscosity and coagulation. Among the collagens this technique has only been applied previously to ground native hide collagen[4,5]. Its application in the present work to soluble collagen, allows the conditions of electrophoresis to be extended to include a wider range of pH and electrolyte solutions than is otherwise attainable for this material.

EXPERIMENTAL

The microelectrophoresis cell (Fig. 1) comprised a glass capillary U-tube, with ground and polished flats in the observation zone, mounted vertically, with silver-silver chloride electrodes sealed in ground glass cones, and held in corresponding sockets.

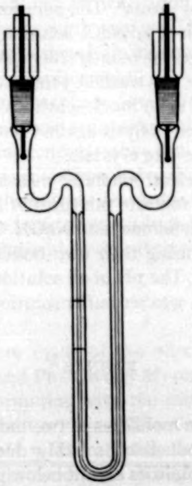

Fig. 1. Microelectrophoresis cell.

Diffusion of products of electrolysis from the electrode compartments was hindered by double bends between the electrodes and the capillary. Observation was through a horizontal microscope, with a × 20 objective and × 10 eyepiece with graticule, calibrated against a stage micrometer. Illumination was through the substage condenser, in contact with the capillary, and at as wide an aperture as possible to minimise the depth of focus. Particles were observed in the central plane of the capillary at a distance of 0.146 the bore diameter from the front, to eliminate the effect of electro-osmotic flow[6], using the previously calibrated fine adjustment of the microscope. The standard correction of HENRY[7] was applied. In measuring the bore diameter it was found advantageous to fill the capillary with a liquid (*e.g.* carbon tetrachloride) of a refractive index near that of the glass, rather than with water, to minimise errors due to optical distortion.

A power pack was used to provide a stable potential, usually of about 110 V. The field strength was calculated from the applied voltage and the resistance of the cell when containing solutions of known conductance; direct calculation from the dimensions of the cell gave a similar but less accurate result. Particle movements were timed to 0.1 sec, with current reversal between measurements, and the mean calculated from at least twenty observations. Most measurements were made between 19° and 21° and were corrected to 20° for viscosity changes by Stokes' Law. The viscosity of the suspensions and collagen solutions used was within 1 % of that of pure water.

The protein was adsorbed on paraffin oil droplets of 1–3 μ diameter, prepared in suspension by a steam-jet method similar to that of DOUGLAS[8] but using superheated steam. Mobilities were measured at surface saturation as shown by the values remaining constant on increasing the protein concentration.

Soluble collagen was prepared from rat tail tendon (of the "August" or "Marshall"-strain, 120–150 g weight), by dissolution in 0.01 *M* acetic acid, followed by purification by a method similar to that of NODA[9]. The solution was filtered through a No. 1 sinter, precipitated, firstly with 5 % NaCl solution, and secondly and thirdly by dialysis against repeated changes of 0.02 *M* NaH_2PO_4; and each time washed and redissolved. The final precipitate was washed 3 times with 0.1 *M* NaCl and with water and redissolved in 0.01 *M* acetic acid. Stock solutions were preserved at 4°, or quickly frozen at — 12°. This material on dialysis against phosphate buffer gave a flocculent mass of the characteristic needle-like crystals.

The final suspensions for electrophoretic measurement, were all of 0.10 ionic strength, and buffered with appropriate mixtures of NaCl, HCl, Na acetate, glycine, Tris (tris (hydroxymethyl) methylamine) and NaOH. The effects of higher valent ions were studied in some instances using their own buffer capacity and maintaining the ionic strength at 0.10 with NaCl. The pH of all solutions was determined immediately before measurement, and for the weaker buffer solutions after the measurements also.

RESULTS

Mobility and pH

The graph (Fig. 2) shows the mobilities of two batches of soluble collagen. Above pH 11.8 mobilities were irreproducible probably due to partial degradation. Both batches have almost identical mobilities except below pH 3, where there is a difference of about 6 %. Specific effects of the lower valent buffer ions used appear small; in regions of overlap there is little difference in mobility, and a smooth curve, without breaks, may be drawn through all the results.

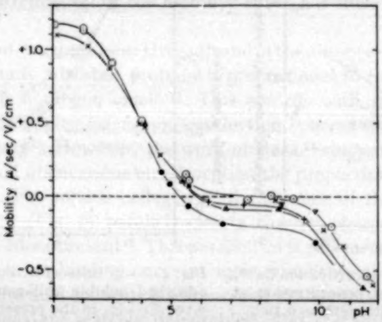

Fig. 2. Microelectrophoretic mobility of adsorbed soluble collagen: Ionic strength = 0.10, 2 batches (⊙) and (×), in the presence of 0.01 *M* phosphate △—△; citrate; (●); +—+ pyrophosphate; ▽—▽ oxalate.

The isoelectric zone is long and flat, between pH 7 and 8.5. The isoelectric point is consequently very sensitive to even small effects of added ions, and is best represented as pH 7.7 ± 0.8.

The influence of di- and tri-valent anions

Mobilities were also determined in solutions containing citrate, phosphate, pyrophosphate, and oxalate anions, all 0.01 *M*, with additional NaCl or appropriate monovalent buffer to bring the ionic strength to 0.10. The results are plotted in Fig. 2. Addition of these anions caused negative shifts in mobility of up to about $0.1 \cdot 10^{-4}$ cm^2 sec^{-1} V^{-1}; in order of decreasing effectiveness:

Citrate > phosphate > pyrophosphate > oxalate; oxalate having very little effect. Correspondingly the isoelectric point was shifted to considerably more acid values. In solutions of the same concentration the isoelectric points were: citrate, 5.3 ± 0.1; phosphate, 5.8 ± 0.2; pyrophosphate, about 5.9.

In the case of citrate, mobilities were determined at a range of concentrations at constant pH (6.7) and the same ionic strength (0.10), and are plotted in Fig. 3. The mobility increment is almost linear at lower citrate concentrations but decreases at higher values.

The influence of divalent cations

Similar determinations were made of the effect of added divalent metal ions (Mg^{++}, Ca^{++}, Cu^{++}, all 0.01 *M* and Pb^{++}, 0.005 *M*) on the mobility, in buffer solutions chosen to minimise complex formation with the other ions present. The only buffer used in the presence of Pb^{++} and Ca^{++} was HCl–NaCl, and hydrolysis restricted the experimental range to below pH 5. The alkaline buffers used for solutions of Mg^{++} and Ca^{++} were Tris and OH^-, in conditions of negligible complex formation or hydrolysis.

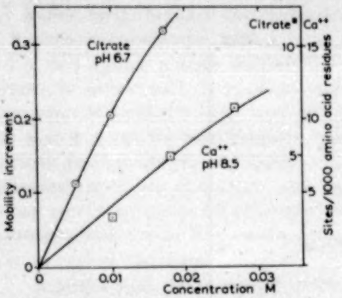

Fig. 3. Mobility increment (positive for Ca^{++}, negative for citrate) against concentration at constant pH and Ionic strength = 0.10.

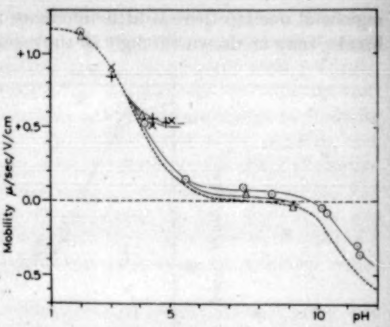

Fig. 4. ---, microelectrophoretic mobility of adsorbed soluble collagen: Ionic strength = 0.10; ⊙—⊙, in the presence of 0.01 *M* Ca^{++}; △—△, Mg^{++} 0.01 *M*; ×—×, Cu^{++} 0.01 *M*; +—+, 0.005 *M* Pb^{++}.

The results are plotted in Fig. 4. Above pH 3, with the possible exception of Mg^{++}, the mobilities are all shifted to more positive values. The effects of Pb^{++} and Cu^{++} are sharp and pronounced, in spite of the relatively short pH range, whilst the effect of Ca^{++} appears to reach a plateau at pH 6–7 and then to increase only gradually. The isoelectric point is correspondingly shifted to higher values, and in the 0.01 *M* Ca^{++} solution used is at pH 9.7 ± 0.2. Results for a sequence of calcium concentrations at constant pH (8.5) and ionic strength are plotted in Fig. 3. The mobility increment is almost linear with Ca^{++} concentration up to about 0.03 *M*.

DISCUSSION

The similarity of the mobilities of free and adsorbed proteins is well established in many instances[10], and it is of interest to compare the present measurements as far as possible with those in free solution. Published mobilities of soluble collagen are at scattered pHs, and large differences of temperature or of the buffer solutions used make precise comparison difficult[2, 3, 14] although no large discrepancies are apparent. The most extensive and also the most comparable determinations are those of BROWN AND KELLY[1] for rat skin soluble collagen, which presumably has an amino acid composition similar to rat tail tendon[13], in acetate buffers between pH 3.55 and 4.86. These results are at a lower temperature (10°) and ionic strength (0.05) than the present series, but parallel them. Moreover their single point at an ionic strength of 0.10, at pH 4.12, agrees with the present value when corrected for viscosity changes to the lower temperature ($0.35 \cdot 10^{-4}$ cm^2 sec^{-1} V^{-1}).

The isoelectric point of the present results (pH 7.7 ± 0.8) can be compared with those of insoluble collagens. Particular interest attaches to the agreement with values determined microelectrophoretically for untreated hide collagen of pH 7[4] and pH 7.8[5]. Similar values have in fact been found by a variety of methods: pH 7.0[16]; pH 7.6–7.8[15]; and pH 7.55–8.15[17]. A much closer relationship between soluble and insoluble collagens is therefore shown than indicated by the previous determination of the isoelectric point of soluble collagen by BROWN AND KELLY, who extrapolated their mobility determinations linearly, to obtain an isoelectric point of pH 5.8. Neglect of the flattening-off of the mobility curve has thus caused an error of about 2 pH units.

In solutions of constant ionic strength and in the absence of other ionic interactions, the mobility of many adsorbed proteins is proportional to the net protein charge due to equilibria with hydrogen ions[10, 11]. This accords with the simple Smoluchowski equation, which is applicable to non-conducting spheres the size of the oil droplets of the present study[19]. However, the work of BULL[11] suggests that owing to the possibility of preferred orientations on adsorption the proportionality constant may vary with the protein. For soluble collagen in the absence of the higher valent ions the pH-mobility curve (Fig. 2) parallels closely the net charge caused by titration of the amino acid residues present[13]. This parallelism is shown very clearly by comparison with the hydrogen ion binding curve at ionic strength 0.5 of hide collagen[18], which has a similar amino acid content. Differences of H^+ binding by collagen in 0.1 M and 0.5 M salt solutions are scarcely detectable[12], and the agreement with the present work is shown quantitatively by the apparent pK values. BOWES AND KENTEN[18] obtained a pK for the carboxyl groups, titrated from about pH 5.5 downwards, of 3.5, whilst that shown by the "half titration point" of the present results is 3.7. The same flat region between pH 5.5 and pH 9.5 is again indicated by both techniques. This agreement together with the lack of specific effects on the mobility of the lower valent buffer ions used, shows that these ions are bound only slightly, if at all, to the protein.

In contrast to the small effects of the lower valent ions, certain of the higher valent ions studied have considerable effects on the mobility. The specificity of these effects suggests that they are due to binding of the ions at sites on the protein. If the proportionality between charge and mobility still applies it is possible to calculate the number of ions of a given charge, required to be bound to cause the observed mobility shifts. The proportionality constant is conveniently derived from the mobility increment due to titration of the carboxyl groups, of which the amino acid and probable amide link content[13] show there to be 83 titratable groups for each thousand amino acid residues. Increasing displacement of the mobility curves thus corresponds to increasing degree of binding, in which order the anions are:

Oxalate < pyrophosphate < phosphate < citrate; and the cations:

$$Mg^{++} < Ca^{++} < Pb^{++} \approx Cu^{++}$$

For citrate and calcium ions the numerical results are plotted on the right hand ordinate of Fig. 3.

The decreasing binding of the divalent cations shown at lower pHs indicates that carboxyl or imidazole sites are involved. Binding of lead at imidazole sites is supported by work by other techniques with insoluble collagen[20] and gelatin[22]. Binding of calcium at carboxyl sites is supported by work on hide collagen[21], although the experimental conditions were not well defined, which showed a large amount of binding at high calcium concentrations, decreasing greatly below pH 3.5. If all carboxyl groups are accessible the results of Fig. 3 yield an intrinsic association constant, ($\log K_{Ca} = 0.57$), very close to that of an isolated carboxyl group in, for example, acetic acid[23].

The sites of anion binding presumably involve cationic amino or guanidinium groups, and the pH effects are probably caused by varying ionization of the small ion rather than of the protein.

ACKNOWLEDGEMENTS

The author thanks Professor W. V. MAYNEORD for his interest and encouragement; Dr. W. ANDERSON for helpful criticism; and Mr. D. P. SMITH for the construction of apparatus.

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Short Communications

The nucleoside phosphorylase of pig liver and calf spleen

We have had occasion to prepare nucleoside phosphorylase from pig liver and wish to report on the presence of a contaminant in the material from this source which interferes with the use of the enzyme in the synthesis of inosine from hypoxanthine.

The phosphorolytic cleavage of inosine by enzymes from liver was first demonstrated by KALCKAR[1,2], who showed that the reaction was reversible, the equilibrium favouring the synthesis of inosine:

$$\text{inosine} + \text{phosphate} \rightleftharpoons \text{hypoxanthine} + \alpha\text{-ribose 1-phosphate}$$

For the enzymic testing of synthetic ribose 1-phosphate, we have prepared an enzyme from pig liver following KALCKAR's method of extraction. The enzyme preparation was highly active in bringing about the phosphorolysis of inosine, but confusing results were obtained on incubation of the enzyme with hypoxanthine and either synthetic or natural ribose 1-phosphate. The reaction was followed using KALCKAR's method: after incubation of the enzyme with hypoxanthine and ribose 1-phosphate for varying periods of time, the enzyme was inactivated by heat, and free hypoxanthine was determined by incubation with xanthine oxidase, which has no action on inosine. The uric acid formed was measured by observation of the increase in absorbancy at 290 mμ (Δ_{290}) during the incubation with xanthine oxidase. Synthesis of inosine is indicated by a decrease in the value of Δ_{290} with increasing times of the initial incubation. Using the pig-liver preparation, it was observed that, although the value of Δ_{290} decreased with increase in the time of incubation with the enzyme, the initial value of the absorbancy at zero time after addition of xanthine oxidase increased with increasing time of the initial incubation with the pig-liver enzyme. Since the molar extinction coefficients of hypoxanthine and inosine are approximately equal and extremely low at 290 mμ, the initial absorbancy should remain constant. The progressive increase in the initial value of the absorbancy at 290 mμ suggested that the decrease in Δ_{290} was not due to the formation of inosine. This was proved to be the case by adding orthophosphate and a further quantity of the pig-liver enzyme preparation to a solution of hypoxanthine and ribose 1-phosphate which had been successively incubated with pig-liver enzyme, heated at 100° for 4 min, and then incubated with xanthine oxidase until the absorbancy at 290 mμ remained constant. No further increase in absorbancy at 290 mμ was observed, indicating that no phosphorolysis of inosine was taking place. Careful removal of orthophosphate from both enzyme preparation and ribose 1-phosphate produced no effect on the results obtained. Incubation of hypoxanthine with the pig-liver preparation without addition of ribose 1-phosphate also resulted in an increase in absorbancy at 290 mμ, suggesting that the enzyme was contaminated with xanthine oxidase. This is not unexpected since the xanthine oxidase of pig-liver can be precipitated with ammonium sulphate in the same concentration range as that used by KALCKAR for the preparation of the nucleoside phosphorylase[3].

Since it was clearly undesirable to add an inhibitor of xanthine oxidase during the first incubation, and since further purification of the nucleoside phosphorylase could not be achieved by several of the usual procedures, attention was turned to the nucleoside phosphorylase of calf spleen[4]. This enzyme is highly active in the phosphorolysis of inosine, but had not been tested for its ability to synthesise inosine from hypoxanthine and ribose 1-phosphate[5]. Using the same techniques as with the pig-liver enzyme, experiments showed that the enzyme from calf spleen contained no xanthine oxidase. By varying the concentrations of the components of the incubation mixture, conditions were found in which the formation of inosine readily took place.

To 1.34 mg of the cyclohexylammonium salt of ribose 1-phosphate in 0.5 ml water, 2 drops of a solution of ammoniacal magnesium citrate were added, and the solution was shaken for 10 min. After removal of the precipitate by centrifuging, the supernatant solution was adjusted to pH 7.5 by addition of succinic acid solution, and was incubated with 0.37 mg hypoxanthine and 2 ml of calf-spleen nucleoside phosphorylase (total activity 650 units as measured by the method of PRICE, OTEY AND PLESNER[4]) in 0.1 *M* glycine–acetate buffer at pH 7.5, the volume being made up to 5 ml with buffer. 1-ml samples were withdrawn at appropriate times, heated in a boiling-water bath for 4 min, cooled, diluted to 5 ml with buffer and centrifuged. To a 2-ml sample of the supernatant 0.002 ml xanthine oxidase solution (approx. 200 units/l[6]) was added, the solution was diluted to 5 ml with glycine–acetate buffer and the absorbancy was measured immediately in a cuvette with a light path of 1 cm and at various intervals until the value remained constant. For various time intervals for the initial incubation, the following values for Δ_{290} were obtained: 0.53 (1 min); 0.23 (5 min); 0.04 (20 min); 0.04 (40 min). We consider that the nucleoside phosphorylase of calf spleen is satisfactory for the demonstration of the reversal of the phosphorolysis of inosine under these conditions.

It is known[7] that xanthine oxidase levels are affected by the nutritional level of animals. It seems likely either that in KALCKAR's experiments, the xanthine oxidase of the liver used was fortuitously low, or that in our experiments the level of this enzyme was unusually high. Whichever is the case, we regard it desirable to record our results which suggest that mammalian liver may not be a suitable source of nucleoside phosphorylase when it is desired to observe the reversal of phosphorolysis.

Gifts of natural ribose 1-phosphate from Dr. H. KLENOW and xanthine oxidase from Professor F. BERGEL, F.R.S. and Dr. R. C. BRAY are gratefully acknowledged. The authors are indebted to the British Empire Cancer Campaign for financial support.

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Received November 30th, 1959

Kinetics of H_2O_2 destruction in *Rhodopseudomonas spheroides*: Roles of catalase and other enzymes

Synthesis of catalase is induced in *Rhodopseudomonas spheroides* by aeration[1]. In studying the kinetics of this phenomenon, using air or H_2O_2 as inducer, it has become necessary to know the intracellular concentration of H_2O_2 in a variety of experimental situations. This will depend on the action of catalase and other agents that decompose H_2O_2. At concentrations greater than 1 m*M*, H_2O_2 is destroyed almost exclusively by catalase* in *Rps. spheroides*[2]. It will be shown here that a peroxidase-like activity is important when the concentration of H_2O_2 is less than about 100 μM.

For these experiments, *Rps. spheroides*, strain 2.4.1, was grown anaerobically in the light, as described earlier[1]. Cultures were tested during exponential growth and in the stationary phase. In the range 1–20 m*M*, the destruction of H_2O_2 by *Rps. spheroides* was assayed by HERBERT's iodometric titration method[4]. This method could be applied to H_2O_2 concentrations as low as 30 μM by increasing the volumes of reagents 10-fold and using more dilute thiosulfate in the final titration. In the range 3–100 μM, H_2O_2 was assayed by its photochemical reaction with luminol, as described by DOLIN[5]. The sensitivity of each method was limited by the interfering effects of dense cell suspensions.

The rate of destruction of H_2O_2 by *Rps. spheroides* depends on the total activity of all peroxide-destroying agents and on the rate at which H_2O_2 can enter the cells. Permeation of H_2O_2 becomes rate-limiting in cells of very high catalase content; the over-all reaction with *Rps. spheroides* then obeys first-order kinetics[2] described by equation (1)

$$-\frac{d[H_2O_2]}{dt} = 0.13\varrho[H_2O_2] \tag{1}$$

where ϱ is the cell-suspension density in mg (dry cell mass)/ml and t is expressed in seconds. If the rate of peroxide disappearance ($-d[H_2O_2]/dt$) is observed to be much less than 0.13 ϱ $[H_2O_2]$, it can be assumed that intracellular reactions, and not permeation, are rate limiting. Extracellular and intracellular peroxide concentrations are then nearly equal.

The permeability problem can be approached by treating the cells with toluene; this treatment destroys permeability barriers toward H_2O_2 without changing the catalase activity. Such cells could not be used in the luminol assay, however, because they produced a large and variable "blank" signal. The luminol assay was therefore restricted to cases in which $-d[H_2O_2]/dt$ was less than 0.04 ϱ $[H_2O_2]$("permeation error" less than about 25 %).

Fig. 1 shows the rate of disappearance of H_2O_2 as a function of its concentration in the presence of *Rps. spheroides* taken from a culture in exponential growth. The ordinate has been normalized to a cell suspension density of 1 mg/ml. Curve A, for peroxide concentrations up to 10 m*M*, has the first-order form appropriate to the action of catalase[3]: $-d[H_2O_2]/dt = k'_1e[H_2O_2]$, where e is the concentration of catalase and k'_1 is a rate constant. In this range of peroxide concentrations the effect of agents other than catalase is nil. Curve B ($[H_2O_2] < 300\ \mu M$) is described accurately by equation (2)

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* The catalase of *Rps. spheroides* has been purified; its molecular weight, specific activity, and absorption spectrum have been determined[9].

$$-\frac{d[H_2O_2]}{dt} = k'_1e[H_2O_2] + \frac{V[H_2O_2]}{K_m + [H_2O_2]}\,; \tag{2}$$

in this range the contribution of catalase ($k'_1e[H_2O_2]$) is shown separately by the dashed line. The term $V[H_2O_2]/(K_m + [H_2O_2])$ is an expression of the MICHAELIS-MENTEN kinetics displayed by most enzymes, including peroxidases. For stationary-phase cells the results are the same except for the magnitudes of k'_1e and V. Values of k'_1e, V, and K_m are shown for growing and stationary-phase cells (suspension density, 1 mg/ml) in Table I. For *Rps. spheroides* catalase, $k'_1 = 53\ \mu M^{-1}\ \text{sec}^{-1}$ and the molecular weight is 230,000 (see ref. 3). The values in Table I for k'_1e then give a catalase content of 0.0015 % of the dry cell mass for cells in exponential growth and 0.0026% for stationary-phase cells. At very low peroxide concentrations ($[H_2O_2] \ll K_m$) Equation (2) becomes

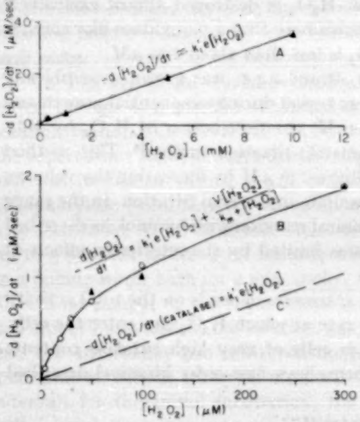

Fig. 1. Kinetics of H_2O_2 destruction by *Rps. spheroides* taken from a culture in exponential anaerobic growth. The ordinate is normalized to a cell-suspension density of 1 mg (dry cell mass)/ml. Curve B is a graph of Equation (2), with $k'_1e = 0.0035\ \text{sec}^{-1}$, $V = 0.91\ \mu M\ \text{sec}^{-1}$, and $K_m = 28\ \mu M$. The term $k'_1e[H_2O_2]$ represents catalase activity, plotted separately as Curve C. The other term, $V[H_2O_2]/(K_m + [H_2O_2])$, represents a peroxidase-like activity. When H_2O_2 exceeds 1 mM this second term is much smaller than the first ("peroxidase" activity ≪ catalase activity). Equation (2) is then approximated by $-d[H_2O_2]/dt = k'_1e[H_2O_2]$ and has the form of Curve A (*cf.* Curve C). ▲, H_2O_2 assayed iodometrically; ○, by the luminol method.

$$-\frac{d[H_2O_2]}{dt} = \left(k'_1 + \frac{V}{K_m}\right)[H_2O_2]\,.$$

and for cells in exponential growth the "peroxidase" activity is nearly 10 times as great as the catalase activity ($V/K_m = 0.032\ \text{sec}^{-1}$, as compared with $0.0035\ \text{sec}^{-1}$ for k'_1e). For stationary-phase cells, with $[H_2O_2] \ll K_m$, "peroxidase" activity is about 3 times as great as the catalase activity ($V/K_m = 0.020\ \text{sec}^{-1}$ and $k'_1 = 0.006\ \text{sec}^{-1}$).

TABLE I

CONSTANTS IN EQUATION (2) DESCRIBING THE DESTRUCTION OF H_2O_2 BY *Rps. spheroides*

Cell-suspension density, 1 mg (dry mass)/ml.

	k'_1 *(sec⁻¹)*	*V (μM sec⁻¹)*	K_m *(μM)*
Cells in exponential growth	0.0035	0.91	28
Stationary-phase cells	0.006	0.56	28

All the H_2O_2-destroying activity was abolished by boiling the cells for 3 min. Catalase and "peroxidase" activities could be separated cleanly by treatment with toluene and sodium azide, respectively. Exposure to toluene abolished the "peroxidase" activity without affecting the catalase, whereas $10^{-4}\,M$ azide at pH 7 inactivated the catalase without influencing the "peroxidase" activity. The latter was inhibited 32% by $10^{-3}\,M$ azide and 83% by $10^{-2}\,M$ azide; these values are appropriate for peroxidases containing heme[6]. A trichloroacetic acid extract of *Rps. spheroides*, prepared as described by VERNON[7], showed negligible peroxidase activity with guiacol and cytochrome *c* as hydrogen donors. Aeration or addition of H_2O_2, sufficient to induce a 10-fold increase in catalase content during 1 h, did not alter the "peroxidase" activity of the initial cells. Thus it is possible to induce wide variations in the relative magnitudes of catalase and "peroxidase" activities in *Rps. spheroides*.

In studies of the effects of H_2O_2 (*e.g.*, in radiobiology), catalase is generally assayed by methods in which the H_2O_2 concentration is millimolar or higher. The destruction of H_2O_2 at much lower concentrations is then predicted from this assay. Such an extrapolation, from the kinetics of peroxide destruction at high concentrations of H_2O_2 to the kinetics at low concentrations, is not justifiable. With *Rps. spheroides*, and perhaps with many other systems, the extrapolation would be grossly in error.

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Received November 28th, 1959

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* Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

Biochim. Biophys. Acta, 40 (1960) 165–167

Obtention du substrat du lysozyme contenu dans la paroi ectoplasmique d'*Eberthella typhi*

Nous avons précédemment montré[1] que la paroi ectoplasmique de *Salmonella* pathogènes pour l'homme, contient un substrat du lysozyme, qui parait—au moins partiellement—responsable de sa rigidité puisque l'activité de l'enzyme se manifeste par sa totale dissolution. Ce substrat, dont on a pu penser qu'il pouvait s'identifier avec l'antigène somatique[2], n'est accessible au lysozyme que si les bactéries ont subi au préalable un traitement approprié dont l'action paraît être d'arracher des constituants pariétaux superficiels riches en lipides. Nous avons cherché à isoler ce substrat, à le purifier et à l'identifier.

La bactérie étudiée est la souche O-901 d'*Eberthella typhi*, que l'on sait exempte d'antigène Vi, peu riche en antigène H et dont la constitution antigénique O est IX, XII. Elle est cultivée à 37° en milieu agité et oxygéné contenant 2 % d'hydrolysat de caséine et 1 % de glucose. La récolte est faite au bout de 24 h par centrifugation, le culot est remis en suspension en tampon phosphate 0.066 M de pH 7.2. Cette suspension est ajustée de telle manière que diluée 25 fois, elle ait une extinction de 0.97 à 720 mμ, puis elle est répartie à raison de 20 ml en ampoules de 25 ml que l'on scelle.

La méthode choisie pour obtenir les parois ectoplasmiques fait appel à une digestion par la trypsine. Par ce procédé en effet, on peut espérer se débarrasser au mieux des fractions protéiques sans modifier les constituants de nature polysaccharidique, parmi lesquels doit se trouver le substrat du lysozyme. Cependant, pour obtenir une bonne dissolution du protoplasme des bactéries et modifier suffisamment la paroi pour que celle-ci puisse être par la suite solubilisée sans que l'on fasse appel à un traitement violent, il est nécessaire de chauffer les bactéries avant de leur faire subir l'action de la trypsine. Cette opération est réalisée en plongeant pendant 10 min les ampoules scellées, dans de l'eau bouillante. On centrifuge, les bactéries sont remises en suspension dans le même tampon, l'extinction est ajustée à 0.97 à 720 mμ puis on ajoute une solution à 5 mg/ml de trypsine très pure de façon à obtenir une concentration finale de 0.5 mg/ml. On incube à 37° pendant 12 h. Les parois récoltées par centrifugation, remises en suspension dans le même tampon, sont soumises quatre fois encore à une digestion trypsique identique. Après ces opérations, le microscope électronique permet de contrôler que le protoplasme a entièrement disparu et qu'il ne persiste plus que les parois isolées. Celles-ci sont entièrement détruites si on les soumet à l'action du lysozyme en tampon trihydroxyaminométhane en présence d'acide éthylène-diamine-tétracétique[1]. En faisant agir dans un volume de 1 ml pendant 2 h, 1 mg de lysozyme sur 21.5 mg de parois sèches, on constate une augmentation du pouvoir réducteur correspondant à 0.194 mg de glucose, ce qui correspond à 7.9 % du pouvoir réducteur total libéré par l'hydrolyse acide.

L'étape suivante consiste à obtenir la dissolution des parois en faisant appel à un traitement aussi peu traumatisant que possible. Dans ce but, on ajoute à la suspension de parois dialysée contre de l'eau distillée et ajustée à l'extinction de 0.97 à 720 mμ, un dixième de son volume de Teepol commercial (solution aqueuse à 21 % du sel de sodium d'alcoylsulfates secondaires). Cette substance tensioactive de pH neutre suffit à détruire entièrement la structure des parois trypsinées dont les constituants passent en solution à l'exception d'une fraction très riche en lipides que l'on écarte par centrifugation.

Il convient alors d'éliminer le Teepol. On y parvient, en le précipitant par le $BaCl_2$, mais les constituants hydrolysables par le lysozyme se trouvent également précipités. Après centrifugation, le culot est agité en présence d'Amberlite XE-66B qui, fixant le baryum, fait passer le Teepol en solution sous la forme acide. Une extraction par l'éthanol permet de se débarrasser de celui-ci. L'insoluble qui contient le substrat du lysozyme est soigneusement lavé à l'éthanol puis traité par l'acide éthylène-diamine-tétracétique. Une fraction sur laquelle le lysozyme est sans action se dissout, et le substrat du lysozyme demeure insoluble sous forme de complexe barytique. Il représente 3.7 % du poids sec des parois, soit 0.64 % du poids sec des bactéries. Son insolubilité n'empêche pas le lysozyme d'agir; celui-ci fait passer en solution 24 % des glucides totaux titrés après hydrolyse acide.

Ce substrat contient 6.1 % d'azote, 0.49 % de phosphore et 11.3 % de baryum. Il est de nature glucidolipidopolypeptidique comme l'antigène somatique. La fraction lipidique représente 15.4 % du poids total dont 10.7 % de lipides très fermement liés, extractibles par le chloroforme, ne sont libérés qu'après hydrolyse acide prolongée. La fraction polypeptidique représente entre 36.3 (dosage des acides aminés libérés par la méthode de KEMBLE ET MAC PHERSON[3] après hydrolyse acide) et 54 % (dosage des acides aminés par la méthode colorimétrique à la ninhydrine après hydrolyse acide). La fraction glucidique exprimée en glucose est de 9.13 % quand elle est estimée par le pouvoir réducteur après hydrolyse acide (dosage par la méthode de SOMOGYI[4]) et de 13.9 % quand elle l'est par la méthode à l'orcinol (méthode de TILLMANS ET PHILIPPI modifiée par RIMINGTON[5]). En réalité, comme cette fraction contient beaucoup d'oses aminés (10 % du poids total exprimé en glucosamine), et que ceux-ci ne sont totalement libérés qu'après une hydrolyse par HCl 6 *N* pendant 8 h à 100° (méthode de BELCMER *et al.*, modifiée par MONTREUIL ET SCHEPPLER[6]), ce traitement brutal détruit beaucoup d'autres glucides et il est vraisemblable que les chiffres précédents sont inférieurs à la réalité.

Dans la fraction polysaccharidique, on a identifié par chromatographie sur papier, la glucosamine et l'acide muramique. Les autres constituants s'ils existent, ne doivent se trouver qu'en très faible quantité.

Dans la fraction polypeptidique, on a identifié l'acide diaminopimélique, l'acide glutamique, l'acide aspartique, la lysine, le glycocolle et l'alanine.

Il résulte de cette composition que le substrat du lysozyme ne parait pas identique à l'antigène somatique puisque la composition de la fraction polysaccharidique est très différente. En revanche, le fait que celle-ci soit riche en hexoses aminés et en acide muramique cadre bien avec ce que l'on sait de la spécificité du lysozyme[7]. De plus, cette composition, ainsi que celle de la fraction polypeptidique, correspond tout-à-fait à celle prêtée par E. WORK[8] au "constituant fondamental de la structure pariétale", de qui dépendraient les propriétés mécaniques des parois et notamment la rigidité. Mais l'existence d'une fraction lipidique solidement liée à ces constituants est assez inattendue.

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Reçu le 27 décembre 1959

Secondary gasation of heme proteins and biological N_2-fixation

Elsewhere[1] we have presented data on the solubility of N_2, H_2, and of A in aqueous legoglobin from soybean-root nodules, as well as in aqueous horse myoglobin and in horse hemoglobin. Here we wish to discuss the implications of these data and related information with respect to the problem of the initial steps in symbiotic N_2-fixation.

It has been established, mainly by VIRTANEN[2], that the capacity of leguminous root nodules to fix atmospheric N_2 goes roughly parallel to the concentration of the heme proteins found in the nodules. We prefer the term legoglobins for these heme proteins rather than leghemoglobin[3] since it is more consistent with the present terminology for the quite similar protein myoglobin. HAMILTON, SHUG AND WILSON[4] have reported an apparent oxidation of ferrous heme iron in root nodule extracts as a result of passing N_2 gas into the extract. These observations imply a possible interaction between N_2 and legoglobins, but are not conclusive in part for reasons discussed below.

We have found a weak interaction between N_2 and Lb, as well as one for Mb and for Hb, in harmony with the earlier discovery of VAN SLYKE *et al.*[5] that oxygenated Hb takes up N_2 to a small extent at 1 atm. The present results may be summarized by stating that the standard free energy of N_2 gas uptake by the heme proteins, ΔF°_u, at 293°K lies within the narrow range of $+ 2.0_0$ to $+ 2.7_0$ kcal/mole, depending on the protein and its oxidation state. This means that at an N_2 pressure of 1 atm only about 1 % of the heme sites are occupied, if we assume one N_2 per heme. Such a weak interaction will be called secondary gasation, partly because it occurs whether primary gasation (*e.g.* oxygenation) has taken place or not. In spite of its weakness, though, secondary gasation seems to be specifically associated with the heme group or possibly with a metal cation, since the non-heme, non-metallic proteins investigated[5,6] so far show no affinity for N_2.

If we suppose that the first step in biological fixation of the N_2 molecule is its attachment to a specific reaction site on some other molecule "X", we must conclude that legoglobin is particularly well suited to play the role of "X". The standard free energy barrier for uptake of N_2 on $LbFe^{+2}$ (2.0_0 kcal/mole) is small relative to other probable barriers[7] in the eventual reduction of N_2 to NH_3; and is quite small relative to the limit of about 20 kcal/mole which reaction-rate theory places on feasible elementary steps of thermal reactions at room temperature. Because of the noble-gas character of N_2, it is difficult to conceive of any aqueous molecule "X" that would have more than a few percent of its sites occupied by N_2 at room temperature and pressure. Hence the total number of available sites per molecule of "X" must be large or the concentration of "X" must be larger than that of enzymes in usual biological systems. We estimate from our yields in the work cited above that the concentration of Lb in root nodules is about $1 \cdot 10^{-4}\,M$ at the height of N_2-fixing capacity, *i.e.* $1 \cdot 10^{-6}\,M$ in N_2-occupied sites at 1 atm. This is a remarkably high value, made possible by the relatively low molecular weight (20,000) and the high solubility of legoglobins. Local concentrations of Lb within the root cell-bacterial complex are likely to be even higher.

Legoglobin, with its Fe^{+2} (or Fe^{+3}) group, also offers the possibility of furnishing

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Abbreviations: Mb, myoglobin; Hb, hemoglobin; Lb, legoglobin.

a reduction (or oxidation) potential just at the point where attachment of N_2 probably occurs, analogous to the probable action of cytochrome a_3 on the O_2 molecule. The redox properties of legoglobin are under investigation in this laboratory. It is interesting that the reduced state of Lb, LbFe^{+2}, has a somewhat greater N_2 affinity than the oxidized state, LbFe^{+3}, a situation more favorable for the reduction of N_2 in the presence of LbFe^{+2} than for its oxidation in the presence of LbFe^{+3}. Oxidation affects myoglobin in a similar way, in harmony with other similarities; but with hemoglobin, the relative affinities of oxidized and reduced states are reversed.

It may be wondered if legoglobin alone is capable of reducing N_2. In our gas-desorption measurements we occasionally observed some slow conversion of LbFe^{+2} to LbFe^{+3} in the presence of N_2, reminiscent of the experiment by HAMILTON, SHUG AND WILSON[4]. However, the same behavior also was (non-reproducibly) observed in the presence of A and of H_2. This leads us to think that, in the absence of O_2 ($<$ 30 p.p.m.) ferrolegoglobin is capable of a disproportionation reaction of the type hinted at by VIRTANEN *et al.*[2], obscuring a possible parallel reaction with N_2. Ascorbic acid prevented the above conversion; and it is noteworthy that ascorbic acid is abundant in root nodules[8]. However, ascorbic acid also lowered somewhat the apparent affinity of LbFe^{+2} for N_2 ($\Delta F^\circ_u = +2.4_2$ kcal/mole), an effect that might be associated with errors in ΔF°_u due to traces of O_2 which ascorbic acid would clean up.

The above facts make it reasonable to hypothesize that a secondary attachment of N_2 to the heme group in legoglobin is the first step in symbiotic N_2-fixation.

We appreciate the support for this work through a grant from the Herman Frasch Foundation and through a Woodrow Wilson National Fellowship for one us (R.G.M.).

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Received November 21st, 1959

Biochim. Biophys. Acta, 40 (1960) 170–171

Photo-oxidation of yeast enolase

Kinetic studies have suggested that histidine residues are involved in both the catalytic action and the metal binding of yeast enolase[1–3]. In order to obtain further evidence for the participation of histidine in the activity of this enzyme, we have subjected it to photo-oxidation in the presence of methylene blue, and attempted

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to determine quantitatively the changes in the protein which are associated with the loss of enzymic activity during the oxidation.

A reaction mixture consisting of 4.0 mg/ml yeast enolase and 0.06 mg/ml methylene blue in 0.05 *M* potassium phosphate buffer, pH 7.1, was irradiated with a 500-W light bulb at a distance of about 10 cm. The samples were maintained at 25–30° in a water bath. At several times aliquots were removed, assayed for activity as described previously[3], and freed of methylene blue by treatment with charcoal. The tryptophan content of the samples was determined colorimetrically[4] on the intact protein. The samples were then hydrolyzed in 6 *N* HCl, and the histidine and tyrosine contents were determined chemically[5,6] and by microbiological assay. Methionine was determined by microbiological assay.

The results of one experiment are summarized in Table I. Only histidine and tryptophan are oxidized to a significant extent during the inactivation of the enzyme. Since the only amino acids which are rapidly destroyed by methylene blue photo-oxidation are histidine, tryptophan, tyrosine, methionine, and cysteine[7], and since enolase contains no cysteine[8], the loss in activity in this case must be due to the destruction of histidine and/or tryptophan residues.

TABLE I

AMINO ACID CONTENT OF PHOTO-OXIDIZED ENOLASE

The values for the amino acid content of the native enzyme are based on data of Malmström *et al*[8].

Oxidation time (min)	*Activity (%)*	*Histidine (moles/mole enolase)*	*Tyrosine (moles/mole enolase)*	*Tryptophan (moles/mole enolase)*	*Methionine (moles/mole enolase)*
0	100	14.0	15.0	5.0	8.0
5	87	14.0	15.4	4.9	9.0
20	58	12.0	13.2	4.5	5.4
40	13	9.5	14.7	3.8	7.4
58	1	6.3	15.3	2.5	7.9

TABLE II

ANALYSIS OF PHOTO-OXIDIZED ENOLASE IN THE ULTRACENTRIFUGE

Activity (%)	*Protein concentration* (mg/ml)*	S_{20} *(sec × 10^{13})*	*Apparent diffusion coefficient at 20° (cm²/sec × 10^{6})*
100**	1.8	5.68	1.01
60	1.5		1.37
27	1.8	6.32	1.57

* Determined spectrophotometrically[9].

** The sample was subjected to exactly the same treatment as the oxidized protein with the exception that methylene blue was omitted.

The oxidized enolase was subjected to physical tests to determine whether photo-oxidation affected the tertiary structure of the enzyme. Although viscosity measurements conducted under suboptimal conditions of protein concentration and flow time showed no significant change over a range of 0 to 90% inactivation, sedimentation measurements gave evidence of structural changes during photooxidation.

Prior to measurement of the sedimentation coefficient in the Spinco Model E Ultracentrifuge, the samples were dialyzed against several changes of 1 M KCl in 0.05 M potassium phosphate buffer, pH 7.0, to remove the methylene blue, and then dialyzed against buffer alone to remove the KCl.

The results of the ultracentrifuge experiments are collected in Table II. The apparent diffusion coefficient was calculated from the ultracentrifuge patterns according to the equation[10]

$$D_{\mathrm{app}} = \frac{1}{4\pi t}\left(\frac{A}{B}\right)^2$$

(The term correcting for the effect of the centrifugal field on different parts of the boundary $(1 - \omega^2 st)$ was found to be insignificant and was thus omitted.) We used D_{app} as a measure of the broadening of the Schlieren image of the boundary and interpreted the boundary spreading as a crude measure of heterogeneity of the protein sample.

Photo-oxidation of yeast enolase thus causes complete loss of activity after the destruction of 8 histidine residues and 2–3 tryptophan residues. This rather extensive oxidation is accompanied by significant changes in the structure of the protein as indicated by the increase in both the sedimentation coefficient and the apparent diffusion coefficient.

Only in instances where the chemical modification of a single group in the enzyme leads to complete loss of activity without altering other parameters is there justification for implicating the modified group in the active site of the enzyme. The data obtained in this study are clearly not subject to this kind of interpretation and earlier conclusions as to the involvement of histidine in the active site of enolase[3] should be re-evaluated.

MALMSTRÖM has recently proposed that 12 of the 14 histidine residues in enolase are involved in the tertiary structure of the native protein[8]. Our findings possibly lend some support to this proposal, since the oxidation of 8 histidine groups involved in such bonding might well lead to inactivation of the enzyme.

This work was supported by a research grant RG-5573 from the National Institutes of Health, U.S. Public Health Service. We are grateful to Dr. R. J. SIRNY and Miss C. LEWIS, Department of Biochemistry, Oklahoma State University, for carrying out the microbiological assays.

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Received November 18th, 1959

Preliminary Notes

Enzymic formation of glyoxylic acid from γ-hydroxyglutamic acid

Of the many glutamic acid derivatives which have recently been discovered in plant extracts, γ-hydroxyglutamic acid is unique in that it has been implicated in the metabolism of both plants and animals. VIRTANEN AND HIETALA[1] detected it in and isolated it from the green parts of *Phlox decussata*. Isotopic studies *in vivo* with mammals[2–4] have suggested that this amino acid is an intermediate in the degradation of hydroxyproline; experiments[5,6] *in vitro* have shown that γ-HG can be formed by oxidation of γ-hydroxyglutamic semialdehyde. However, no information is available on further enzymic reactions utilizing γ-HG as substrate. This communication reports the presence in rat liver of an enzyme system which catalyzes the breakdown of γ-HG with the formation of glyoxylic acid.

The γ-HG used in these studies, synthesized by a modification of the procedure of BENOITON AND BOUTHILLIER[7], had m.p. 169–171°. (Found for $C_5H_9O_5N$: C, 36.87; H, 5.56; N, 8.55. Calc.: C, 36.81; H, 5.52; N, 8.59). On paper chromatograms in three different solvent systems, the compound migrates as one single spot. Homogenates of fresh or frozen rat liver catalyze the conversion of γ-HG to a product having an aldehydic group. The reaction is followed by oxidizing the phenylhydrazone of the aldehyde in the presence of excess phenylhydrazine with $K_3Fe(CN)_6$ and measuring the colored formazan at 540 mμ. Table I illustrates the enzymic nature of this reaction. β-Hydroxyglutamic acid, β,γ-dihydroxyglutamic acid, and threonine will not replace γ-HG, nor will various hydroxylated dicarboxylic acids including malic, citric, isocitric, and β-hydroxy-β-methylglutaric acids. Preincubation of dialyzed KCl-

TABLE I

ENZYMIC GLYOXYLATE FORMATION FROM γ-HYDROXYGLUTAMIC ACID

The complete system contained 300 μmoles of Tris buffer, pH 8.1, 5 μmoles of synthetic γ-HG (neutralized with KOH), and 0.3 ml (3 mg protein) of a 10% homogenate of fresh rat liver in 0.5 *M* KCl. Water was added to give a final volume of 3 ml. After incubation at 37° under N_2 for the times indicated, the reaction mixtures were deproteinized with 1 ml 12% metaphosphoric acid and 1-ml aliquots removed for the determination of glyoxylic acid.

Expt.	*System*	*Glyoxylic acid formed* (μmole)*
1	Complete, zero time	0
2	Complete, 15 min incubation	0.06
3	Complete, 30 min incubation	0.14
4	Complete, 60 min incubation	0.25
5	Expt. 4 without γ-HG	0
6	Expt. 4 without rat-liver homogenate	0
7	Expt. 4 with heat-denatured rat-liver homogenate	0

* Estimated by an adaptation of the colorimetric procedure of D. N. KRAMER *et al.*[10].

Abbreviations: γ-HG, γ-hydroxyglutamic acid; Tris, tris(hydroxymethyl)aminomethane; DPNH, reduced diphosphopyridine nucleotide.

ethanol extracts[8] of rat liver with pyridoxal 5-phosphate stimulates the enzymic reaction approximately 10–20 %. Treatment of the enzyme with carbonyl reagents also suggests that pyridoxal 5-phosphate is involved. The following degrees of inhibition are observed when a preparation of the enzyme, purified about 19-fold, is preincubated with the compound listed (at 0.01 *M* concentration): semicarbazide, 72 %; hydroxylamine, 100 %; phenylhydrazine, 100 %; sodium bisulfite, 71 %.

The enzyme system has been purified from dialyzed KCl–ethanol extracts approximately 19-fold by acetone and ammonium sulfate fractionation and heat treatment. Use of this partially purified system results in the accumulation of several micromoles of the metabolite which allows it to be identified as glyoxylic acid in the following ways. (1) The 2,4-dinitrophenylhydrazone of the enzymic product has an R_F value which is identical on Whatman No. 1 filter paper in several solvent systems with the synthetically prepared 2,4-dinitrophenylhydrazone of glyoxylic acid (m.p., 196–197°). (2) The elution pattern of the metabolite from a Dowex 1-X10 column (acetate phase) with 4 *M* acetic acid is essentially the same as that for an equivalent amount of pure glyoxylic acid[9]. (3) DPNH is oxidized by the isolated metabolite in the presence of very highly purified glyoxylic acid reductase*. The aldehyde formed from γ-HG was chromatographed on a Dowex 1-X10 column (acetate phase), eluted, and the eluate lyophilized and neutralized. The oxidation of DPNH, measured by the decrease in absorption at 340 mμ and catalyzed by glyoxylic acid reductase, depends upon the addition of the isolated aldehyde and occurs at a rate comparable to that for an equivalent amount of known glyoxylate.

In addition to glyoxylic acid, alanine has been shown by chromatography on Whatman No. 1 filter paper in three different solvent systems to be a product formed in the complete enzyme system. Presumably, these two metabolites could arise by direct cleavage of γ-HG. If this is true, such a reaction would represent a unique example of an aldol-type cleavage of an amino acid in which the hydroxyl group is not on a carbon atom immediately adjacent to the carbon atom bearing the amino group.

This work was supported by grants from the Horace H. Rackham School of Graduate Studies, The University of Michigan, and the U.S. Public Health Service (Grant A-3718).

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Received March 14th, 1960

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* Kindly furnished by Dr. Zelitch, New Haven, Conn.

The respiratory activity and respiratory control of sarcosomes isolated from the thoracic muscle of the housefly

Although it is known that the thoracic muscles of flying insects are rich in cytochrome[1] and in large mitochondria (sarcosomes)[2,3], and have *in vivo* a high respiratory activity which is controlled by the muscular activity[4], it has proved difficult to isolate sarcosomes with the properties to be expected from these findings. Oxidative phosphorylation was not demonstrated until 1953 (ref. 5, 6), while respiratory control (*i.e.* dependence of the rate of respiration on the concentration of ADP) has only very recently been achieved[7,8]. The values for the P:O ratio and the Q_{O_2} (μl O_2/mg sarcosomal protein/h) which have been reported are much lower than those expected from the activity *in vivo*[9-11].

In the present report it is shown that, if sarcosomes isolated from the thoracic muscle of the housefly *Musca domestica*, essentially by the method of LEWIS AND SLATER[5], are tested in the presence of a supernatant fraction, high Q_{O_2}'s, P:O ratios and respiratory control can be measured. A typical experiment with succinate as substrate is shown in Fig. 1 and average values are given in Table I. The addition of the supernatant fraction caused a large increase in the respiratory rate and in the P:O ratio, with the establishment of respiratory control. In other experiments, Q_{O_2}'s of 695*, 486, and 458 were obtained under similar conditions with α-glycerol phosphate (0.05 *M*), DPNH (0.05 *M*) and pyruvate (0.02 *M*) + malate (0.002 *M*), respectively, as substrate.

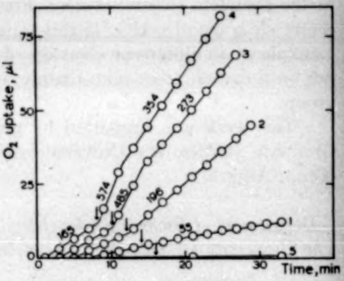

Fig. 1. The effect of supernatant fraction on the respiration of sarcosomes isolated from housefly thoracic muscle. 200 thoraces from flies 8–18 days old were gently pounded in a mortar with 5 ml 0.25 *M* sucrose, 1 m*M* EDTA, pH 7.4, and the brei filtered through muslin. The filtrate was centrifuged at 150 × *g* for 3 min and the supernatant centrifuged for 8 min at 3000 × *g*. The sedimented sarcosomes were suspended in 4 ml isolation medium. The supernatant fraction contained 3.34 mg protein/ml. The reaction mixture contained 15 m*M* KCl, 2 m*M* EDTA, 5 m*M* $MgCl_2$, 0.05 *M* Tris-acetate buffer, 0.03 *M* potassium phosphate, 0.1 m*M* ATP, 0.06 *M* succinate, 0.63 mg sarcosomal protein. The pH was 7.5, the reaction volume 1 ml and the temp. 25°. At the arrow, 3 µmoles ADP were added from the side bulb. Curve 1, no further addition; curves 2, 3 and 4, with 0.1, 0.2 and 0.3 ml supernatant fraction, respectively; curve 5, with 0.3 ml supernatant fraction without sarcosomes. The values given over straight parts of the curves are Q_{O_2}'s (μl O_2/mg sarcosomal protein/h).

Similar results were obtained by working with the suspension obtained by filtering the muscle brei through muslin. The mean P:O ratio (number of experiments in brackets) of these homogenates were: succinate, 2.03 (10); α-ketoglutarate (in the presence of 0.02 *M* malonate), 2.27 (3); glutamate, 2.30 (3). The P:O ratios were

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Abbreviations: ADP, ATP, adenosine di- and triphosphate; EDTA, ethylenediaminetetraacetic acid; DPNH, reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

* This is the average rate over 30 min. The initial Q_{O_2} was 783.

unaffected by varying the concentration of added adenine nucleotide between zero and 1.8 m*M* (contrast ref. 12). In the presence of ADP, the rate of respiration was increased 11-fold by adding inorganic phosphate, with either succinate or α-ketoglutarate as substrate.

TABLE I

RESPIRATORY ACTIVITY, RESPIRATORY CONTROL AND OXIDATIVE PHOSPHORYLATION WITH SARCOSOMES ISOLATED FROM HOUSEFLY THORACIC MUSCLE, WITH SUCCINATE AS SUBSTRATE

The values given are means with the number of experiments in brackets.

Addition	*Respiratory activity* µl O_2/mg sarcosomal protein/h*	*Respiratory-control index***	*P:O**
None	58 (7)	1.0 (3)	0.59 (3)
Serum albumin (2 %)	49 (4)	1.0 (1)	1.21 (2)
0.1 ml supernatant fraction	270 (2)	1.87 (3)	1.88 (2)
0.2 ml supernatant fraction	538 (2)	3.83 (3)	2.20 (2)
0.3 ml supernatant fraction	625 (3)	3.51 (5)	2.25 (2)

* Measured as in Fig. 1, but with 0.03 *M* glucose + hexokinase in place of ADP. P:O ratios determined by method of Lewis and Slater[5].

** (Q_{O_2} in presence of ADP): (Q_{O_2} in presence of 0.1 m*M* ATP).

Further studies are required to elucidate the mechanism of action of the supernatant fraction. Its effect is abolished by heating for 5 min at 100°. In any case, it is clear that the isolated sarcosomes are capable of a high activity in the Krebs cycle, and that this oxidation is accompanied by oxidative phosphorylation and is under control of the ADP concentration. The respiratory rates measured in the presence of ADP, which are much higher than those previously reported, approach those found *in vivo*[4,9-11].

This work was supported in part by a grant from the Life Insurance Medical Research Fund.

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Received March 12th, 1960

Incorporation of leucine into microsomal albumin by microsomes and pH-5 enzymes from normal rat liver

The uptake of [^{14}C]amino acids into microsomal protein by cell-free systems has been a subject of interest since the study of Keller and Zamecnik[1]. Peters[2] demonstrated, using chick-liver slices, that the microsome is the main site of serum–albumin synthesis. Campbell *et al.*[3] further noted [^{14}C]amino acid uptake into microsomal albumin in a system consisting of microsomes and cell sap of the regenerating rat liver. The present experiments were undertaken to elucidate the participation of amino-acid-activating enzymes, soluble RNA and the microsome system in albumin synthesis by normal liver cells.

Rat-liver pH-5 enzymes[*] and microsomes, prepared by the method of Keller and Zamecnik[1], were incubated with uniformly labeled [^{14}C]-L-leucine at 30° for 30 min with some modifications according to the method of Littlefield *et al.*[7]. After incubation, Peter's desoxycholate extraction[2] of the microsome fraction was carried out, and microsomal albumin was then precipitated from the dialyzed deoxycholate-soluble fraction by the addition of a suitable amount of rabbit antiserum[**] against the rat serum albumin[***]. The identification of the precipitate was made by the Ouchterlony's gel diffusion[9] and immuno-electrophoretic methods. The protein fractions were then subjected to purification and their specific activities were determined as reported in our previous papers [5,10,11]. The specific activity of microsomal albumin was corrected by the factor for the antigen-antibody ratio according to the method of Heidelberger[8,10,11].

The results are summarized in Table I. It was of interest that GTP was indispensable for the incorporation of [^{14}C]leucine into microsomal albumin, together with ATP and an ATP-generating system. In agreement with the results of experiments by Peters[2] with liver slices, the specific activity of microsomal albumin was considerably higher than that of the total microsome desoxycholate-soluble protein fraction, indicative of active albumin synthesis by this system. The requirement of pH-5 enzymes for the incorporation was demonstrated by the use of washed microsomes (Table I, B). The pre-incubation of the reaction mixture with a small amount of RNase resulted in a remarkable decrease in the incorporation (Table I, C). The fact that the pre-incubation of pH 5 enzymes with RNase followed by the precipitation at pH 4.7 resulted in marked inhibition of [^{14}C]leucine uptake into microsomal albumin, suggested the participation of soluble RNA in albumin synthesis (Table I, D). The further addition of the pH 5-supernatant fraction[6] increased the incorporation into both protein fractions, and its lability to heat suggested that it is enzymic in nature. The result is in agreement with recent investigations of Zamecnik *et al.*[4] and Grossi *et al.*[12]. The 2,4-dinitrofluorobenzene treatment of the pooled microsomal albumin and ribonucleoprotein by the method of Sanger[13] indicated that [^{14}C]-leucine was not bound to the free amino groups of the proteins.

Abbreviations: RNA, ribonucleic acid; ATP, adenosine triphosphate; GTP, guanosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

[*] Isoelectric precipitation of pH 5 enzyme was made once at pH 4.7.

[**] Antiserum was absorbed with rat serum globulin and the titer was determined by Heidelberger's method[8] prior to use.

[***] Rat serum albumin was purified by $(NH_4)_2SO_4$ fractionation, followed by starch zone electrophoresis.

Biochim. Biophys. Acta, 40 (1960) 178–179

TABLE I

INCORPORATION OF $[^{14}C]$LEUCINE INTO ALBUMIN AND RIBONUCLEOPROTEIN OF RAT LIVER MICROSOMES

	Specific activity (counts/min/mg)	
	Albumin	*Ribonucleoprotein*
(A) Complete system	2500 (1390)*	3390
minus ATP	19	150
minus phosphocreatine, creatine kinase	220	394
minus GTP	40	340
(B) Complete system	1220 (505)*	1615
minus pH-5 enzymes	292	189
(C) Complete system	2700 (1040)*	3260
plus RNase	290	133
(D) Complete system	2020 (575)*	2220
with RNase-treated pH-5 enzymes	850	191

* The specific activities of total microsome deoxycholate-soluble proteins are reported in parentheses.

(A) The complete system: 0.5 mmole sucrose, 10 μmoles $MgCl_2$, 100 μmoles KCl, 20 μmoles $KHCO_3$, 50 μmoles Tris buffer (pH 7.9), 2 μmoles ATP, 0.5 μmole GTP, 40 μmoles phosphocreatine, 0.17 μmole of $[^{14}C]$leucine (0.5 μC), 0.1 mg creatine kinase, microsomes (12 mg protein). pH-5 enzymes (8 mg protein) in total volume of 2 ml. Incubation at 30° for 30 min.

(B) The same conditions as in (A), except that once-washed microsomes were used and 0.08 μmole $[^{14}C]$leucine was added.

(C) The incubation mixture without $[^{14}C]$leucine was pre-incubated without or with 0.5 μg RNase/2 ml for 10 min at 30°.

(D) pH-5 enzymes pre-incubated without or with RNase (5 μg/ml) for 30 min at 10° were used.

The results would seem to raise the possibility that albumin synthesis in liver cells could be formulated through amino-acid-activating enzymes, soluble RNA and the microsome system in analogy to the hemoglobin synthesis by reticulocytes reported by SCHWEET *et al.*[14].

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Received February 22nd, 1960

Early effect of estradiol on amino acid penetration in rabbit uterus

The stimulation of protein synthesis by estrogen in uterine tissue has been observed to follow the early initial water and electrolyte changes[1]. Estrogenic stimulation of the incorporation of radioactive substrates into uterine tissue components has required several hours pretreatment *in vivo* or long incubation in tissue culture[2]. These apparent differences have lead to separate experimental consideration of electrolyte and protein processes. However, RIGGS[3] has shown in the Ehrlich ascites tumor cell that the penetration of amino acids into the cell, the earliest step in cellular protein synthesis, is intimately related to potassium metabolism. With the demonstration of a correlation between electrolyte metabolism and cellular penetration of amino acids it becomes pertinent to consider the earliest effect of estrogen on amino acid penetration into uterine tissue. Amino acid transport has been examined with the non-utilizable amino acid [1-^{14}C]AIB and the association of uterine tissue with estrogen has been observed with [16-^{14}C]estradiol-17β.

Immature, ovariectomized New Zealand white rabbits were used in three types of experiments: estradiol stimulation *in vivo* followed by AIB penetration *in vitro*, estradiol AIB incubation together *in vitro*, and radioactive estradiol penetration *in vivo* and *in vitro*. Rabbits were anesthetized and control horns removed to incubation flasks containing buffer and AIB. After the intravenous injection of estradiol the contralateral experimental horns were removed at various times and incubated with AIB in a manner similar to the controls. In the experiments *in vitro* the rabbits were sacrificed and the control horns placed in incubation flasks with buffer and AIB while the flasks with the contralateral experimental horns had estradiol in addition. After the incubations the tissues and media were assayed for AIB as previously described[4,5]. Estrogen-penetration studies *in vivo* were carried out by injecting rabbits intravenously with radioactive estradiol, removing uterine tissue at various

TABLE I

EFFECT OF TREATMENT WITH ESTRADIOL ON ABILITY OF UTERUS TO CONCENTRATE AIB

Each uterine horn was incubated at 38° with continous oxygenation in 20 ml of Krebs-Ringer phosphate buffer, pH 7.4. AIB of specific activity $1.7 \cdot 10^9$ counts/min/mmole was at a concentration of $8.78 \cdot 10^{-4}$ M. Estradiol was at level of 0.5 μg/ml. AIB was determined as counts/min/ml of medium or total tissue water, from which the concentrating ability of the tissue relative to the medium was calculated. The figures reported show the per cent increase in the ability of the estrogen-treated uteri to concentrate AIB as compared to the controls. The figures in parenthesis are the number of experiments.

	Incubation time	
	30 min	*60 min*
Estradiol *in vivo*		
30 min		131 ± 10 (3)
60 min		159 ± 1 (2)
120 min		195 ± 14 (2)
Estradiol *in vitro*	0.46 ± 6.7 (19)	7.3 ± 9.6 (11)

Abbreviation: AIB, α-aminoisobutyric acid.

times and obtaining a terminal blood sample. Estrogen penetration *in vitro* was observed by incubating the uterine horns in buffer containing radioactive estradiol. The tissues, blood plasma and media were analyzed by determining the ^{14}C content. All radioactivity determinations were corrected to infinite thinness.

TABLE II

[16-^{14}C]ESTRADIOL UPTAKE BY UTERUS

In vivo experiments. Rabbits were injected with 181 μg [16-^{14}C]estradiol-17β, 1963 counts/min/μg. Uterine horns were removed after being exposed to the circulating estrogen for 30 and 60 min. *In vitro* experiments. Uterine horns were pooled and incubated for 30 and 60 min in 50 ml of Krebs-Ringer phosphate buffer containing 0.605 μg/ml of radioactive estradiol. Plasma was prepared for ^{14}C determination by plating an aliquot; incubation media were diluted with 10% egg albumen prior to plating. Tissues were ground with 10 vol. water and aliquots of the total homogenates were plated for counting.

Reaction time (min)	*In vivo*		*In vitro*	
	counts/min/g uterus	*counts/min/ml plasma*	*counts/min/g uterus*	*counts/min/ml medium*
30	204 ± 20 (2)		7,590 ± 50 (3)	985
60	340 ± 4 (2)	346 ± 32	11,276 ± 310 (3)	925

The results show that estradiol when injected *in vivo* has an immediate effect on amino acid penetration, which is concomitant with the earliest time when electrolyte shifts may be observed. The early effect *in vivo* is accompanied by an association in uterine tissue of radioactivity which was derived from estradiol. The stimulation *in vivo* of penetration of amino acids by estradiol is not found in analogous experiments *in vitro*. The failure *in vitro* cannot be attributed to inability of estradiol to penetrate uterine tissue since the experiments *in vitro* show that uterine tissue can concentrate the estradiol from the medium.

These experiments demonstrate that the very early effects of estradiol on the uterus include a stimulation of the first step in protein synthesis, the cellular penetration of amino acids. The dependence of the stimulation on the presence of the organism suggests that estradiol is converted *in vivo* to some other active form.

This work was supported in part by a grant to the Department of Obstetrics and Gynecology by the Josiah Macy Foundation.

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Received March 7th, 1960

Reversible inhibition of aldolase by ferricyanide

The inhibitory action of ferricyanide on the aerobic and anaerobic glycolysis of some types of tumour cells[1–3] has now been found to be due to an inactivation of aldolase[*].

Homogenates of the Walker 256 rat carcinoma, Crocker mouse-ascites sarcoma 180, Ehrlich mouse-ascites carcinoma, rat-brain cortex and rat-kidney medulla were prepared in 0.125 M KCl, with the use of a Potter-Elvehjem homogenizer, and centrifuged at 3000 rev./min for 5 min. The supernatant (S_1) was used as such or after centrifugation at 0–4° at 100,000 × g for 60 min (S_2). The glycolytic activity of these supernatants was measured at 25° under air in a modified medium of LePage[4] containing, in a final volume of 1.5 ml, 25 mM potassium phosphate buffer (pH 7.4), 1.4 mM ATP, 0.4 mM DPN, 40 mM nicotinamide, 4 mM $MgCl_2$ and 5 mM fructose 6-phosphate or fructose 1,6-diphosphate. Fluoride and pyruvate were omitted. Final osmolarity was adjusted to 0.25 with KCl. Lactic acid formation was determined colorimetrically[5].

Aldolase, glyceraldehyde 3-phosphate dehydrogenase and a mixture of glycerophosphate dehydrogenase and triosephosphate isomerase were obtained from Boehringer. Aldolase and glyceraldehyde 3-phosphate dehydrogenase were assayed spectrophotometrically by the methods used by Wu and Racker[6], in the case of aldolase with excess glycerophosphate dehydrogenase and triosephosphate isomerase. Fructose 1,6-diphosphate was estimated spectrophotometrically[7].

Two tumours (Walker and Crocker) with a ferricyanide-sensitive glycolysis were used. Glycolysis in both the low- and high-speed supernatants (S_1 and S_2) from these tumours was inhibited completely by 2–5 mM $K_3Fe(CN)_6$. This was the case with either fructose 6-phosphate or fructose 1,6-diphosphate as substrate. A small excess

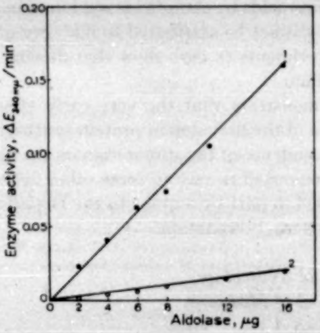

Fig. 1. Effect of ferricyanide on muscle aldolase activity, measured spectrophotometrically[6]. The activities were calculated from the linear decrease of the absorbancy at 340 mμ, measured in a medium containing 0.05 M Tris buffer (pH 7.4), 2 mM fructose 1,6-diphosphate, 0.1 mM DPNH, with excess glycerophosphate dehydrogenase and triose phosphate isomerase, and the indicated amounts of aldolase. Curve 1, control; curve 2, with 0.5 mM $K_3Fe(CN)_6$.

Abbreviations: ATP, adenosine triphosphate; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

[*] In connection with the stimulation of the hexose monophosphate oxidation by ferricyanide previously reported[8], attention is called to the fact that a correction should be made to the values given for μ-atoms glucose carbon oxidized. All values should be divided by a factor 2.75. This correction does not affect the relative rates of oxidation of C-1 and C-6.

of cysteine, which reduced the $K_3Fe(CN)_6$, partially reversed this inhibition (cf. the disappearance of the ferricyanide effect in intact cells after washing. Fructose 1,6-diphosphatase activity was negligible in the preparations used and the phosphorylation of fructose 6-phosphate to fructose 1,6-diphosphate with ATP, studied in the presence of 1 m*M* iodoacetate, was not affected by 5 m*M* $K_3Fe(CN)_6$.

Concentrations of ferricyanide as low as 0.5 m*M* suppressed the activity of commercial crystalline muscle aldolase almost completely (Fig. 1). The inhibition was completely reversed by 5 m*M* cysteine.

Commercial purified glyceraldehyde 3-phosphate dehydrogenase was practically unaffected by 1 m*M* $K_3Fe(CN)_6$.

Addition of aldolase to a cell-free tumour preparation, in which glycolysis was abolished by ferricyanide, restored the glycolytic activity (Fig. 2). This is not due to removal of ferricyanide, since the ferricyanide concentration at the end of the incubation was still sufficient to inhibit glycolysis in the absence of added aldolase. Glyceraldehyde 3-phosphate dehydrogenase added to the inhibited system did not relieve the inhibition.

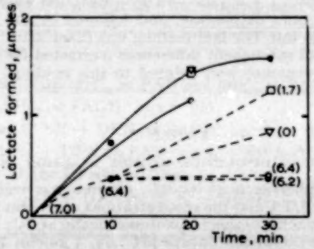

Fig. 2. Effect of ferricyanide on the production of lactic acid from fructose 1,6-diphosphate by a cell-free preparation (S_1) of the Crocker sarcoma 180. See text for reaction conditions. Broken lines represent experiments with 5 m*M* $K_3Fe(CN)_6$. 0.8 mg aldolase (□), 0.8 mg glyceraldehyde 3-phosphate dehydrogenase (○), or 15 μmoles cysteine (▽), respectively, added at zero time in the control experiments and after 10 min in those with ferricyanide. ●, no additions. The values in brackets are the amounts of ferricyanide (μmoles) found at the times indicated.

Although intact normal tissue characterized by high glycolysis[2] and Ehrlich ascites cells[3] are not sensitive to $K_3Fe(CN)_6$, the aldolase activity of cell-free preparations from Ehrlich tumour, rat-brain cortex and rat-kidney medulla was sensitive to ferricyanide. The difference between ferricyanide-susceptible and non-susceptible cells must probably be ascribed to different permeabilities of the cell wall to ferricyanide.

I wish to thank Prof. E. C. SLATER for his interest and advice.

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Received March 4th, 1960

On the reaction mechanism of lipoyl dehydrogenase

It has been shown that lipoyl dehydrogenase from pig heart is a flavoprotein[1–3] identical with STRAUB[4] diaphorase. Further studies have shown that in catalysis, the flavin is functioning between the oxidized and semiquinoid levels[5], with only one interconversion of FAD and FADH˙ per catalytic cycle. The fate of the other electron involved has been clarified by the finding that a second molecule of pyridine nucleotide and the conversion of a protein disulphide linkage to a protein dithiol are involved in the reaction. The reduction of lip S_2 by DPNH shows a lag period which occurs to varying extents with some commercial samples of DPNH but not with others and hence was attributed to the presence of an inhibitor[3]. Fig. 1 shows that this lag period

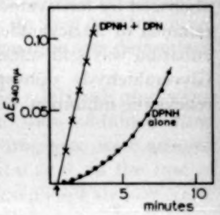

Fig. 1. The effect of DPN on the reduction of lip S_2 by DPNH. The reactions were carried out at 25° in a volume of 3 ml. Each cell (1 cm light path) contained 2500 µmoles citrate (pH 5.65), 3 µmoles EDTA, 2 mg serum albumin, 2 µmoles DL-lip S_2 and 0.6 µmole DPNH (freshly made Sigma 98 %) ± 0.3 µmole DPN. Reaction was begun by addition of 1.4 µg pure diaphorase, and followed by the decrease in absorbancy at 340 mµ. The first reading was taken after 30 sec (indicated by arrow); all subsequent differences (corrected for blank rates in the absence of enzyme) were related to this reading.

TABLE I

SUBSTRATE-INDUCED DISULPHIDE–DITHIOL REACTION IN DIAPHORASE

In the experiments with diaphorase (0.35 mg/ml), enzyme was incubated at 0° with 0.03 *M* phosphate pH 6.3, $3 \cdot 10^{-3}$ *M* EDTA and the concentrations of reactants shown. Samples of 0.01–0.03 ml were withdrawn to test activity spectrophotometrically at 25°. In the lip S_2 assay each cell contained in a vol. of 3 ml, 2500 µmoles citrate pH 5.65, 3 µmoles EDTA, 2 mg bovine serum albumin, 2 µmoles DL-lip S_2, 0.3 µmole DPNH, 0.3 µmole DPN. Absorbancy changes were followed at 340 mµ after the addition of enzyme. The DCIP assays were performed as described previously[3]. In the experiments with KGD, enzyme (1.15 mg/ml) was incubated at 0° with 0.03 *M* phosphate pH 7.4, $3 \cdot 10^{-3}$ *M* EDTA and the concentrations of reactants shown. KGD was prepared as described previously[2]. Samples of 0.01–0.03 ml were taken for assays as described above.

	Relative activity	
	with LipS₂	*with DCIP*
Diaphorase at 0°	(100)	(100)
Diaphorase incubated with 10^{-3} *M* arsenite	100	100
Diaphorase incubated with $4 \cdot 10^{-4}$ *M* DPNH	100	1000
Diaphorase incubated with $4 \cdot 10^{-4}$ *M* DPNH + 10^{-3} *M* arsenite	17	1400
KGD complex at 0°	(100)	(100)
KGD complex incubated with 10^{-3} *M* arsenite	105	120
KGD complex incubated with $4 \cdot 10^{-5}$ *M* CoASH + $3 \cdot 10^{-3}$ *M* KG*	105	70
KGD complex incubated with $4 \cdot 10^{-5}$ *M* CoASH + $3 \cdot 10^{-3}$ *M* KG + 10^{-3} *M* arsenite*	8	70

* These reaction mixtures also contained 10^{-3} *M* cysteine.

Abbreviations: CoASH, coenzyme A; DPN, DPN˙ and DPNH, oxidized, half-reduced and reduced diphosphopyridine nucleotide; EDTA, ethylenediaminetetraacetate; FAD, FADH˙ and $FADH_2$, oxidized, semiquinoid and fully reduced flavin-adenine dinucleotide; KG, α-ketoglutarate; KGD, ketoglutarate dehydrogenase complex; lip S_2 and lip $(SH)_2$, oxidized and reduced lipoic acid; TPP, thiamin pyrophosphate.

is due to the production of DPN, which is required as a cofactor in the reaction. The function of a reactive disulphide group in the enzyme has been revealed by studies with arsenite, as shown in Table I. When diaphorase or KGD was incubated with 10^{-3} M arsenite and then diluted 100–300 fold in the assay, inhibition was produced only in the presence of reducing substrate. The activation of the DCIP activity observed with diaphorase was also produced by DPNH alone and hence is clearly a different phenomenon to the inhibition of the lip S_2 activity. The inhibitions were obtained very rapidly and were stable for several hours. After that they reversed slowly as the reducing substrate became exhausted through a slow reaction of the enzyme with O_2. The inhibitions were independent of the order of addition of the arsenite and substrates.

It is clear from the results presented here that arsenite inhibition is not *necessarily* a reaction with enzyme-bound lip$(SH)_2$ as postulated before[6-8, 2] but that an arsenite-sensitive dithiol is involved in lipoyl dehydrogenase, which has been separated from the rest of the KGD complex[2,9], and shown not to contain any bound lipoic acid[2]. In view of the involvement of the flavin at the semiquinoid level[5] and the results presented here it is proposed that the reaction mechanism of lipoyl dehydrogenase may be represented as follows:

$$\text{FAD enz S}_2 + \text{lip (SH)}_2 \rightleftharpoons \text{FAD enz (SH)}_2 + \text{lip S}_2 \tag{1}$$

$$\text{FAD enz (SH)}_2 \rightleftharpoons \text{FADH}^{\cdot}\text{ enz S}^{\cdot}\text{SH} \tag{2}$$

$$\text{FADH}^{\cdot}\text{ enz S}^{\cdot}\text{ SH} + \text{DPN} \rightleftharpoons \text{FADH}^{\cdot}\text{ enz S}_2 + \text{DPN}^{\cdot} + \text{H}^+ \tag{3}$$

$$\text{FADH}^{\cdot}\text{enz S}_2 + \text{DPN} \rightleftharpoons \text{FAD enz S}_2 + \text{DPN}^{\cdot} + \text{H}^+ \tag{4}$$

$$2\ \text{DPN}^{\cdot} + \text{H}^+ \rightleftharpoons \text{DPNH} + \text{DPN} \tag{5}$$

$$\text{Overall:lip (SH)}_2 + \text{DPN} \rightleftharpoons \text{lip S}_2 + \text{DPNH} + \text{H}^+ \tag{6}$$

In this formulation, it is proposed that the sharing of electrons between DPNH and DPN (reaction(5)) may be very much enhanced by the binding of these substances at adjacent groups in the enzyme, so that the near-planar pyridine rings lie closely parallel. This situation, similar to that proposed by GRABE[10] for electron transfer between pyridine nucleotide and flavin, would be expected to result in electron-sharing between the two enzyme-bound pyridine nucleotides.

One of us (C.V.) is an O.E.E.S. senior visiting fellow, on leave of absence from the Laboratory of Physiological Chemistry, University of Amsterdam, working under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.), with financial aid from the Netherlands Organization for the Advancement of Pure Research.

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Received March 2nd, 1960

Intracellular localization of catalase and of some oxidases in rat liver

In rat liver, the intracellular distribution of uricase has been found by several workers to resemble that of acid phosphatase, without, however, being entirely identical with it. The significance to be attached to these findings has been diversely appreciated. While HOGEBOOM *et al.*[1] have favoured the view that the two enzymes belong to the same particles, other authors like THOMSON AND MOSS[2] have preferred to leave the matter open. In this laboratory, acid phosphatase has been found to be associated, together with a number of other acid hydrolases, with a special group of cytoplasmic particles which have been called *lysosomes* (for a review see ref. 3). The general parallelism between uricase and the lysosomal enzymes has been confirmed, but attention has been drawn to the greater homogeneity and higher D_2O-binding capacity of the uricase-containing particles, as possible indications that they may be distinct from the lysosomes[3-5].

Conclusive evidence supporting the latter view has now been obtained by submitting mitochondrial fractions from rat liver to density equilibration in a gradient of glycogen, extending linearly from 0–0.23 g/ml in 0.5 *M* aq. sucrose. In this system, the uricase particles accumulate in the layers immediately below the sedimentation boundary of the glycogen and are almost completely separated from the lysosomes, which are concentrated at the bottom of the tube. The bulk of the mitochondria occupy an intermediate position. Like the lysosomes, the uricase-containing particles are associated with a very small proportion of the total cell nitrogen.

Two other enzymes, catalase and D-amino acid oxidase, have been found to share the main properties of uricase. As already indicated by the investigations of THOMSON AND KLIPFEL[6] on catalase and of PAIGEN[7] on D-amino acid oxidase, they tend to concentrate in the light mitochondrial fraction in fractionations performed according to DE DUVE *et al.*[4]. In addition, they show a higher equilibrium density than the mitochondria and even than the lysosomes in a sucrose–D_2O gradient prepared according to BEAUFAY *et al.*[5], and a lower one in the glycogen–0.5 *M* sucrose gradient described above. Their density–distribution curves do not, however, coincide perfectly with that of uricase in the same systems and it must therefore be concluded that the three enzymes either belong to separate particles showing very similar properties or that they are associated together, but in varying proportion, with a single group of particles. In the latter event, the particles concerned could be an important site of H_2O_2 metabolism, since they would contain two oxidases known to form this compound, in association with the most important enzyme destroying it. Another implication of these findings is that the protection of cellular constituents against H_2O_2, formed, for instance, under the influence of ionizing radiations, may be restricted to very small areas of the liver cell.

In contrast with the oxidases mentioned above, monoamine oxidase shows a true bimodal distribution. The mitochondrial component, which accounts for about 70% of the total activity, accompanies cytochrome oxidase in several types of gradients and must be considered truly mitochondrial. The microsomal component, on the other hand, can be largely separated from the bulk of the microsomal protein, phospholipid and RNA, as well as from several other enzymes present in this fraction (glucose-6-phosphatase, esterase, DPNH-cytochrome *c* reductase, β-glucuronidase,

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Abbreviations: DPNH, reduced diphosphopyridine nucleotide; RNA, ribonucleic acid.

fumarase) by density equilibration in an aqueous sucrose gradient. In this system, monoamine oxidase shows a distinctly lower equilibrium density than the other components. Fumarase, on the other hand, shows an exceptionally high equilibrium density, which depends on the presence of intact RNA.

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Received March 10th, 1960

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The chromatographic behaviour and adenosine triphosphatase activities of the meromyosins

Previous investigations[1] have shown that when L-myosin is chromatographed on diethylaminoethylcellulose the ATPase activities of the eluted fractions vary in a characteristic way. Comparable results have also been obtained in preliminary studies with carboxymethylcellulose[2] and BRAHMS[3] has also reported a fractionation of the ATPase activity. To assess the significance of these indications of the enzymic heterogeneity of chromatographed myosin, L-meromyosin and H-meromyosin have been studied by similar techniques.

H-meromyosin, prepared by the method of SZENT-GYÖRGYI[4] and shown to sediment as a single peak in the ultracentrifuge, was consistently fractionated into two components when applied to a diethylaminoethylcellulose column equilibrated against 0.15 M KCl, 20 mM Tris–HCl, pH 7.6 or 8.2. Of the total eluted material estimated by its absorption at 280 mμ, 12–13 % passed through the column unheld, whereas the main component was eluted as a symmetrical peak at 0.19–0.20 M KCl,

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Abbreviations: ATP, adenosine triphosphate; Tris, tris(hydroxymethyl)aminomethane.

20 m*M* Tris–HCl, pH 7.6. The bulk of the ATPase activity was associated with this peak which consisted of material possessing 230–240 units of specific ATPase activity[*], *i.e.* 10–20 % higher than that of the unchromatographed H-meromyosin. The specific ATPase activity was constant in all fractions except for a falling off in the lower regions of the trailing edge of the peak. It is worth noting that the specific activity of some fractions of L-myosin obtained in the earlier study were comparable to those obtained with chromatographed H-meromyosin.

The average specific ATPase activity of the minor peak was about 30–40 % of that of the original H-meromyosin and assay on individual fractions showed a sharp fall in activity from the front of the peak to the trailing edge, where it was either very low or negligible.

L-meromyosin preparations[4] invariably possessed ATPase activity which, when related to absorption at 280 mµ[*], was 10–20 % of that of the original myosin. These preparations, which gave single peaks in the ultracentrifuge, were resolved into three fractions on diethylaminoethylcellulose. The first fraction represented about 5-12 % of the original L-meromyosin and was not held in diethylaminoethylcellulose in 0.15 *M* KCl, 20 m*M* Tris–HCl, pH 8.2. This peak possessed only slight, barely significant, ATPase activity whereas there was a characteristic pattern of distribution of specific ATPase activity along the second and main component eluted with its peak at 0.21 *M* KCl, 20 m*M* Tris–HCl, pH 8.2. Highest values were obtained in the first fractions of this peak and the ATPase activity dropped sharply to a fairly constant value of about 15-30 units over the remaining two thirds of the peak.

The trypsin digest of myosin possessed about 50 % of the 5′-adenylic deaminase activity of the original myosin; nevertheless none of this enzyme was present in purified L-meromyosin or H-meromyosin.

An interesting feature of the fractionations reported here was that the ribonucleoprotein invariably found associated with myosin preparations[1,5] was concentrated in the third component isolated from L-meromyosin. This was eluted by the stepwise application of 2 *M* KCl, 20 m*M* Tris–HCl, pH 8.2, after previous removal of the ATPase-containing peak. Under similar conditions H-meromyosin preparations yielded no significant amount of nucleoprotein.

It can be concluded from this investigation that the conventional L-meromyosin and H-meromyosin preparations are chromatographically complex. Further the chromatographic behaviour of the ATPase of H-meromyosin is more compatible with that of a homogenous enzyme preparation than are the results with L-myosin. These findings are of significance in interpreting the results of end-group investigations[6], turnover studies of L-meromyosin and H-meromyosin[7,8], and in the localization of myofibrillar proteins by fluorescent antibody techniques using these myosin derivatives[9].

The investigations are being continued to throw light on the sub-unit structure of myosin.

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[*] Unit of specific activity[1]: µg P/5 min/1 ml protein of $E_{280}^{1\,\mathrm{cm}} = 1$; measured at 25°.

[**] This work was done during tenure of an Advanced Research Fellowship from the American Heart Association.

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Received March 19th, 1960

Biochim. Biophys. Acta, 40 (1960) 187–189

On the transfer of labeled amino acids from "S-RNA" to purified ribonucleoprotein particles from rat liver microsomes

The incorporation of labeled amino acids into protein by RNP from rat-liver microsomes is enhanced by an enzyme preparation (S-protein[1]) from the soluble liver fraction[2]. The effect of S-protein is increased by GSH or MEA.

The S-protein preparation used in the present experiments corresponded to the purified "S_{80}-protein" of RENDI *et al.*[2], and it had been treated with charcoal for 1 h at 4°. The absence of ribonucleotides in the preparation was ascertained in the following way. S-protein was prepared from rats, treated with 2 mC of [^{32}P]phosphate for 18 h. Carrier RNA was added to the preparation, and ribonucleotides were isolated by chromatography after hydrolysis. No radioactivity was observed in the nucleotide fractions. The activity of the preparations in amino-acid-dependent isotope exchange between [^{32}P]pyrophosphate and ATP (PP–ATP exchange) was slightly variable, but usually small. As a rule no significant formation of [^{14}C]L-leucylhydroxamate could be demonstrated by paper chromatography[3] after incubation of [^{14}C]L-leucine with ATP and NH_2OH in the presence of S-protein.

RNP were prepared by treating mitochondria-free, rat-liver homogenates (in 0.25 *M* sucrose, 0.5 *M* KCl, 0.01 *M* $MgCl_2$ and 0.035 *M* Tris buffer, pH 7.8) with 0.5 % Lubrol W and 1 % sodium deoxycholate, followed by centrifugation through a layer of medium with higher density[2,4]. The incorporation of labeled amino acids into protein by these particles in the presence of S-protein, MEA, ATP, GTP, PEP and pyruvate kinase was amino-acid specific. The incorporation was not inhibited by the presence of other, unlabeled amino acids in a 10-fold excess. Since the S-protein preparations used in these experiments had a quite low activity of amino acid activation, the possibility was considered that certain amounts of amino-acid-activating enzymes were still present in the particles. By use of prolonged incubation periods (60 min) and incubation samples containing 5–7 mg of protein an amino-acid-dependent PP-ATP exchange, amounting to 3.7 %/mg protein, was actually observed in particles of this kind.

Abbreviations: RNP, ribonucleoprotein particles; RNA, ribonucleic acid; ATP, GTP, triphosphates of adenosine and guanosine, respectively; PEP, phosphoenol pyruvate; GSH, reduced glutathione; MEA, 2-mercaptoethylamine; Tris, tris(hydroxymethyl)aminomethane; PP, [^{32}P]pyrophosphate.

Biochim. Biophys. Acta, 40 (1960) 189–191

In order to get RNP of higher purity, the particles were homogenized with the same combination of detergents and medium as before, and centrifuged a second time by layering. By this treatment the RNA/protein ratio of the particles increased from 0.42 to 0.54. The amino-acid-dependent PP–ATP exchange, measured by a 60-min period, decreased to 1.9 %/mg protein. The incorporation of [^{14}C]L-leucine into protein by the particles in the absence of soluble enzymes decreased by the washing from 90 counts/min/cm^2 to 16 counts/min/cm^2. In the case of the washed particles only a slight enhancement of the incorporation was obtained by the addition of S-protein, even in the presence of MEA or GSH.

When a preparation of a pH-5 enzyme fraction, prelabeled with [^{14}C]L-leucine[5], was incubated with the washed particles in the presence of ATP, GTP, PEP and pyruvate kinase, an incorporation of isotope into protein occurred. The incorporation was not significantly stimulated by MEA or GSH, but an increase by about 20 % was observed, when S-protein was added in combination with one of these reducing agents. When S-RNA, prelabeled with [^{14}C]L-leucine[5] or with a mixture of [^{14}C]-labeled amino acids, was incubated with washed RNP in a system containing ATP, GTP, PEP and pyruvate kinase, no significant isotope incorporation was observed, and no stimulation was obtained with MEA or GSH. In the presence of the pH-5 enzyme fraction, however, the labeled S-RNA-amino acids were readily transferred to the particles and incorporated into protein, even in the presence of a large excess of the corresponding non-labeled amino acids (Table I). The incorporation was stimulated by GSH or MEA (10^{-2} *M*). The pH-5 precipitable fraction could be replaced by S-protein, and also in this case a marked increase in the incorporation was observed in the presence of GSH or MEA. The pH optimum of this reaction was 7.8–8.0. The activity of the S-protein is of a particular interest, since this preparation had a much lower activity of amino-acid-dependent PP–ATP exchange than the pH-5 precipitable fraction.

When washed pH-5 enzyme fractions were preincubated for 45 min at 35° and reprecipitated at pH 5.1, their activity in the transfer systems diminished. At the

TABLE I

TRANSFER OF [^{14}C]-L-LEUCINE FROM S-RNA TO PURIFIED RNP

The incubation tubes contained in 1.5 ml of 0.015 *M* KCl, 0.01 *M* $MgCl_2$, and 0.035 *M* Tris, pH 7.8: 1 µmole ATP, 10 µmoles PEP, 15 µg pyruvate kinase, 0.2 µmole GTP, 0.2 µmole unlabeled L-leucine, 80 µg S-RNA-[^{14}C]-L-leucine, RNP corresponding to 5 mg protein, and the indicated amounts of enzyme preparations and GSH. After 30 min at 35° the reaction was stopped by 2 ml 10 % trichloroacetic acid. The samples were extracted with hot trichloroacetic acid, ethanol and ether and the radioactivity determined by a thin-mica end-window counter.

Enzyme preparation added	*mg protein*	*PP-ATP exchange**	*Transfer of [^{14}C]-L-leucine to RNP; total counts*	
			without GSH	*GSH, 10^{-2} M*
None	—	—	9	10
pH-5 enzyme fraction	2.5	17.7	67	88
S-protein	4.2	5.6	16	48
S-protein from pH-5 fraction	0.9	19.8	34	42

* Exchange was measured by incubating the indicated amounts of the enzyme preparations for 12 min with 6 µmoles ATP, 6 µmoles $MgCl_2$, 4 µmoles [^{32}P]pyrophosphate and 60 µmoles KF[3].

same time a significant, but less pronounced, decrease of their PP–ATP exchange activity was observed. Proteins, non-precipitable at pH 5.1, appeared during the incubation, and from the incubation mixture a nucleotide-free enzyme preparation could be obtained by the same method, which was used for the preparation of the S-protein. The transfer activity per mg protein was considerably higher in this S-protein from the incubated pH-5 fraction than in the S-protein obtained from the pH-5 supernatant[2]. At the same time, however, a higher activity of PP–ATP exchange was observed (Table I).

This work was supported by a grant from the Swedish Cancer Society.

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Received February 26th, 1960

A comment on the pH-dependent dissociation of haemoglobins

It is known that normal adult human haemoglobin (Hb-A) dissociates reversibly both at low pH (5–5.5)[1] and high pH (11–11.2)[2] into units having half the molecular weight of the parent molecule, and evidence has been adduced which indicates that the dissociation is asymmetric with respect to the four peptide chains ($\alpha_2\beta_2$) in the molecule[3,4].

These observations have a bearing on the question of the different resistance of Hb-F (foetal haemoglobin) and Hb-A to denaturation by alkali. Under selected conditions[5] (pH about 12.8) the reaction follows apparent first-order kinetics with $t_{1/2}$ values (*i.e.* time for 50 % conversion to alkaline haematin) at 20° of 11 sec and 1030 sec for Hb-A and Hb-F respectively. A mixture of the two species thus gives a first-order reaction plot consisting of two linear components with widely differing slopes, joined by an intermediate curved region.

Hb-F—if its behaviour is comparable with that of Hb-A—must have dissociated into half-molecules, α_2^{A} and γ_2^{F}, well below this pH, and Hb-A into α_2^{A} and β_2^{A}; the α-chains are reported to be common to both species[6,7]. Since Hb-A is wholly labile to alkali, both the α_2^{A} and β_2^{A} chains must be rapidly denatured. It seems therefore to follow that the alkali resistance of Hb-F is a property of the γ_2^{F} units. Furthermore, if it indeed consists of equal proportions of alkali-labile and alkali-resistant chains, there should be a marked change in slope in its denaturation-rate plot, corresponding to the presence of a resistant portion amounting to 50 % of the total Hb-F, irrespective of

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Abbreviations: Hb-A, normal adult human haemoglobin; Hb-F, human foetal haemoglobin.

any further dissociation that the half-molecules may undergo. This is not in fact observed.

Two possible explanations present themselves: firstly, that Hb-F may not dissociate in the same way as Hb-A; secondly, that the dissociation equilibrium is a slow one, so that under the stated conditions denaturation has occurred before dissociation. In order to investigate the first of these possibilities, cord-blood haemoglobin (75 % Hb-F, 25 % Hb-A) was dialysed against 0.1 M Na_2CO_3 (pH 11.1) and sodium phosphate buffer (pH 11.6, I 0.22) and examined in the ultracentrifuge at a protein concentration of about 0.7 %. Molecular-weight determinations were made by the method of ARCHIBALD[8], according to procedures used by CHARLWOOD[9]. The partial specific volume of haemoglobin was taken as 0.749. At pH 11.1 a $S_{20,w}$ value of 2.42 with an attendant molecular weight of 53,000 was obtained, corresponding evidently to dissociation of the Hb-A portion only. On the other hand, at pH 11.6 (at which the solution fully retains the spectroscopic characteristics of haemoglobin for some days) the $S_{20,w}$ value was 2.20 and the molecular weight 32,500, indicating that complete dissociation had occurred. The dissociation equilibrium of Hb-F is thus shifted towards higher pH compared with Hb-A, but at the pH value at which the denaturation-rate measurements are made, the molecule is nevertheless dissociated.

In order to eliminate complications arising from the possibility that the dissociation process may be slow, and to ensure that the pigment subjected to denaturation was in fact dissociated, the cord-blood haemoglobin was first dialysed for 24 h at 4° against buffers of pH 11.1 to 11.6. The reaction[5] was then followed photometrically (interference filter, maximum transmission 625 mμ). No change in slope apart from the initial section corresponding to the Hb-A component was observed, and there were no significant differences from the normal-rate curve for cord blood haemolysates; extrapolation of the terminal linear part of the plot led as before to an intercept corresponding to 75 % Hb-F in the mixture.

It is difficult, therefore, to escape the conclusion either that the α-chains of Hb-A and Hb-F are not precisely identical with respect to peptide structure or haem-protein linkage, or that other factors may enter into the dissociation at high pH, in particular that it might be symmetrical rather than asymmetrical. If such is in fact the case, the results of a number of hybridisation experiments on human haemoglobins carried out at high pH would be open to re-interpretation[4,10].

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Received February 11th, 1960

FRACTIONATION AND COUNTERCURRENT DISTRIBUTION OF RIBONUCLEIC ACID

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(Received August 12th, 1959)

SUMMARY

1. RNA has been prepared from rat and chicken liver by phenol–water and phenol–naphthalene-1,5-disulphonate (NDS) methods. The RNA prepared by the phenol–NDS method appears to be less degraded than RNA isolated by phenol–water.

2. RNA can be fractionated by addition of 2-butoxyethanol or 2-ethoxyethanol to solutions containing potassium acetate. The most insoluble fraction has the highest proportion of guanine and cytosine, while the fraction with the highest content of adenine and uracil is the most soluble. 2-Ethoxyethanol is better than 2-butoxyethanol for fractionation of RNA prepared by the phenol–NDS method.

3. C.C.D. of RNA and the fractions show that a complex mixture of interacting substances is present.

4. Fraction 1 (phenol–NDS preparation) appears to be the most undegraded RNA but action of ribonuclease can bring about a change in the C.C.D. pattern simulating that of the RNA prepared by the phenol–water method and without altering the base composition of the material.

INTRODUCTION

Evidence for the heterogeneity of ribonucleic acids (RNA) has been produced by several methods. Metabolic heterogeneity was demonstrated by SACKS, HURLEY AND YOUNG[1] by injection of radioactive phosphorus into rats and rabbits. MAGASANIK[2] has discussed results of the examination of base compositions of RNA present in different fractions obtained by differential centrifugation of tissue homogenates. Generally the base composition of the nuclear fraction differed from that of the cytoplasmic fractions and this conclusion has been supported by ELSON, TRENT AND CHARGAFF[3]. DE LAMIRANDE, ALLARD AND CANTERO[4], however, reported considerable variation in the base compositions of all their fractions from rat liver. Fractionation of RNA of ECTEOLA anion exchange resin by BRADLEY AND RICH[5] definitely demonstrated the large number of components in a given preparation and similar results were obtained by MUIRA AND SUZUKI[6] who used Dowex-2 and found fractions of differing molecular weights, but not of base composition. DAVIS AND ALLEN[7] fractionated yeast RNA by using salmine sulphate as a precipitant and releasing RNA by addition of sodium dodecyl sulphate solution, while BROWN, DAVIES, COLTER, LOGAN AND KRITCHEVSKY[8] separated RNA of high molecular weight from Ehrlich ascites tumour cells by fractionation on a column of methylated bovine serum albumin. The present work demonstrates a relatively simple method of separating rat and chicken liver RNA into fractions of different base composition, and a study of the relationship of these fractions to each other and to the original RNA by application of the countercurrent distribution method of CRAIG AND CRAIG[9].

MATERIALS AND METHODS

Solvents

2-Methoxy-, 2-ethoxy- and 2-butoxy-ethanols are available as technical solvents and PORTER[10] reported a purification of these which yielded material having little absorption at 280 mμ if the original solvent had an absorbancy of less than 0.5 at 280 mμ. Frequently the original optical density was much higher than this and, as the method employed by PORTER did not give a sufficiently low O.D. at 260 mμ, a new method for the purification was devised. 4 l of the technical solvent were mixed with 1 g of palladised charcoal (5 %) and a stream of hydrogen was passed into this mixture through a sintered glass bubbler during 2 h. The solvent was filtered into a 3-necked flask for distillation and about 2 g of amidol (2,4-diaminophenol hydrochloride) were added. The solvent was distilled at the pressure of the water pump (approx. 15 mm) in a stream of N_2. A fractionating column 60 cm long was used and an arrangement made to collect fractions for measurements of the O.D. It is usually necessary to collect about 500 ml before the O.D. drops to 0.1 at 260 mμ, but this volume varies from one batch to another. When the O.D. was below 0.1 the solvent was collected until there remained about 100 ml of dark residue in the distillation flask. The solvent collected had an O.D. of 0.02–0.05 at 260 mμ and usually no absorption at 280 mμ. Under these conditions 2-methoxyethanol boils at about 31°, 2-ethoxyethanol at 42° and 2-butoxyethanol at 72°. These solvents may then be kept in dark bottles at room temperature for several weeks without an increase in the O.D. at 260 mμ.

Phosphate solution

K_2HPO_4 was used for making the phosphate solution required for counter current distribution (C.C.D.). No difference in O.D. at 260 mμ was observed when Analar KH_2PO_4 and KOH were used instead. Since K_2HPO_4 contains a variable amount of moisture, a solution based upon specific gravity was used as a standard. K_2HPO_4, sp. gr. 1.320 at 20° was made up by weighing 1320 g K_2HPO_4 and adding 2.5 l distilled water. The solution was filtered, cooled to 20° and adjusted to sp. gr. 1.320 by addition of water (usually 100–200 ml). 33.3 % (v/v) H_3PO_4 was prepared by adding 350 g H_3PO_4 (Analar, sp. gr. 1.75) to 400 g of water. The phosphate (pH 7.5) solution was made by addition of 6 g of 33.3 % H_3PO_4 to every 100 g K_2HPO_4 (sp. gr. 1.320).

Solvent systems

They were made up by weighing and were allowed to remain at 20° overnight before being used.

Solvent system 1: 2-methoxyethanol 740 g, 2-butoxyethanol 360 g, phosphate, pH 7.5, 1600 g, water 1420 g. This mixture produced approximately 1.5 volumes top phase per 1 volume lower phase.

Solvent system 2: 2-ethoxyethanol 3000 g, water 5600 g, ammonium sulphate 2000 g. This system gave nearly equal volumes of each phase.

Counter current distributions

The C.C.D.s were performed with a Quickfit and Quartz fully automatic machine (10 ml upper phase, 11 ml lower phase). RNA (15–30 mg) used for each distribution was first dissolved in water, and the phosphate and organic solvents then added in the correct proportions. After the distribution water was added to each tube to bring the contents into one phase and the O.D. was measured at 260 mμ.

Preparation of RNA

The phenol–water preparations were made essentially as described previously[11] and in addition the following modifications were made; naphthalene-disulphonate (NDS) preparations were made by homogenising the tissue in a volume equal to 8 × weight of the tissue of a solution of 0.5 % naphthalene-1,5-disulphonate disodium salt to every 100 ml of which 0.1 ml 2.5 M K_2HPO_4 had been added to bring the pH to 6.5. The mixture was then stirred and extracted with an equal volume of 90 % (w/w) phenol at 20° and the procedure described before was followed exactly. The yields were the same.

One preparation was made from rat liver which was homogenised in 0.18 % NaCl (equivalent molar concentration of 0.5 % NDS), extracted with phenol and isolated as described previously, and a further preparation was made by homogenising the rat liver with the phenol–water mixture in the Waring blendor and subsequent procedures were the same as in previous preparations.

Fractionation of RNA

Three fractions were obtained by the following method. RNA (3 g) was dissolved by stirring in water (450 ml) with KOAc (9 g). Alterations of the volumes mentioned for a given weight of material will produce a different result. The mixture was centrifuged in a Servall centrifuge at 5000 × g for 30 min at 0° to remove traces of insoluble material. The supernatant liquid was poured off, 2-butoxyethanol (300 ml distilled) was added and the mixture was set aside in the refrigerator overnight. A gelatinous precipitate had separated and was centrifuged off at 700 × *g*. This precipitate constituted fraction 1, and was washed twice with ethanol–water (3:1, v/v) when it separated as a white powder which was sometimes slightly grey on drying. 2-butoxyethanol (150 ml) was added to the clear supernatant fraction from fraction 1 and the mixture was again set aside overnight at 2°. This separation was facilitated if carried out in centrifuge bottles since the precipitate was not apparent although the solution appeared opalescent when shaken. This solution was centrifuged at 700 × *g* for 20 min and the material which remained at the bottom of the tube, after carefully pouring off the supernatant layer, constituted fraction 2. This residue separated as an oil, but when washed with ethanol–water (3:1, v/v) immediately separated as a white powder, which was washed once more with ethanol–water before drying in a vacuum desiccator. To the supernatant solution from fraction 2 ethanol (625 ml) was added and a flocculent precipitate appeared quickly but was allowed to remain overnight before being collected by centrifuging. The final precipitate was washed twice with ethanol–water (3:1) before being dried in a vacuum desiccator and constituted fraction 3. The total recovery of material was about 90%.

Fractionation of fraction 1 with ethoxyethanol

Fraction 1 from rat liver RNA (NDS preparation) (3.0 g) was dissolved in water (300 ml), KOAc (6 g) was added and then 300 ml 2-ethoxyethanol. Precipitation was allowed to proceed overnight at 2°, and the separated material was difficult to centrifuge but was deposited by centrifugation at 5000 × *g* for 45 min. After washing and drying this material weighed 2.2 *g*. The supernatant solution from this precipitate was mixed with 450 ml ethanol and the precipitate which constituted fraction 1/3 was separated by centrifugation, washed and dried (780 mg). The material which separated first was dissolved in water (200 ml), KOAc (4 g) added and 200 ml 2-ethoxyethanol; after standing overnight at 2° the precipitate was separated by centrifugation (5000 × *g* for 40 min), washed and dried and constituted fraction 1/1. Fraction 1/2 was obtained from the supernatant solution by adding of 300 ml ethanol and separation of the insoluble RNA by centrifuging. Rat liver RNA (phenol–water preparation) and chicken liver RNA were separated into 2 fractions only, the first precipitated by 2-ethoxyethanol and the second by ethanol as described for rat liver RNA.

Commercial yeast RNA

"Purification" was carried out by dissolving commercial yeast RNA (2 g) in water (50 ml) adding an equal volume of 2.5 *M* K_2HPO_4 mixed with 2.5 ml 33% H_3PO_4 (1 volume H_3PO_4 sp. gr. 1.75 + 2 volumes H_2O) and 50 ml 2-methoxyethanol. The upper phase, which was dark brown, was separated and dialysed against 4 changes of distilled water when most of the colour disappeared. The contents of the dialysis bag were made up to 2% with respect to KOAc and the RNA precipitated by 2 volumes of ethanol. After centrifuging, washing and drying the yield was 770 mg.

Action of ribonuclease on RNA fraction

Each fraction (50 mg) was dissolved in 4% KOAc solution (25 ml) (fraction 1 dissolved somewhat slowly) and to each solution was added pancreatic ribonuclease (1 mg) in water (25 ml). The mixture was allowed to remain for 2 h at 20° and then shaken vigorously with 50 ml phenol (90% v/v) for 30 min. The mixtures were poured into separating funnels and after 1 h the phenol layer was run off. Ethanol (80 ml) was added to the aqueous layer to precipitate degraded RNA, which was centrifuged off, washed with ethanol–water (3:1) and dried in a vacuum desiccator.

Action of ribonuclease on fraction 1/1 at different times

Fraction 1/1 from a NDS–phenol preparation from rat liver was used and 164 mg were dissolved in 74 ml of a solution of 2% KOAc. Pancreatic ribonuclease (1 mg) in 2 ml water was added, and the mixture was shaken thoroughly and allowed to stand at 20°. 25 ml of solution were removed after 15, 30 and 60 min and in each case added immediately to 25 ml phenol (90%). Each was shaken for 30 min before separating the phenol as described before and precipitating the degraded RNA with ethanol.

Action of ribonuclease and NDS on rat liver RNA

RNA (160 mg) was dissolved in water (75 ml) and KOAc (3 g) was added. This solution was divided into 3 portions of 25 ml each. To the first 25 ml was added 25 ml H_2O, to the second was added 1 mg ribonuclease in 25 ml water and to the third was added 1 mg ribonuclease in 1% NDS (adjusted to pH 7.0 with 2.5 M K_2HPO_4). The 3 solutions were allowed to stand for 20 min, at 20°, 90% phenol (50 ml) added to each and well shaken, the phenol layer was run off from a separating funnel and the RNA precipitated from the aqueous layer by addition of ethanol (80 ml). The insoluble material was centrifuged off, washed and dried as usual.

Analytical methods for phosphorus, $\varepsilon(P)$ (CHARGAFF[12]) and base compositions have been described before[11].

Amino acids were estimated after hydrolysis with 5.8 M HCl for 24 h and separation of the amino acids by 2 way paper chromatography used ethyl methyl ketone–acetic acid–water (3:1:1, v/v/v) for the first way and ethyl methyl ketone–methanol–water–NH_3 solution (sp. gr. 0.88) (3:1:1:0.15, v/v/v/v) the second way. The colours were developed with ninhydrin and estimated as the cadmium complexes, (unpublished).

Ribonuclease was the crystallized commercial pancreatic enzyme (General Biochemicals Inc.).

RESULTS

Fractionation of RNA

The fractions produced by addition of 2-butoxyethanol to solutions of RNA in 2% potassium acetate varied slightly in amount and base composition from one preparation to another. However, the fraction with the greatest proportions of guanine and cytosine always separated first and a higher proportion of fraction 1 was always obtained from preparations made by the phenol–NDS method. The base compositions and yields of the fractions separated from rat-liver RNA prepared by the phenol–water and phenol–NDS methods are shown in Table I.

TABLE I

BASE COMPOSITIONS IN MOLAR PROPORTIONS AND ANALYTICAL FIGURES OF THE FRACTIONS OBTAINED FROM RAT-LIVER RNA BY PRECIPITATION WITH 2-BUTOXYETHANOL

Abbreviations: G, guanine; A, adenine; C, cytosine; U, uracil; P, phosphorus; $\varepsilon(P)$ as defined by CHARGAFF[12].

	G	*A*	*C*	*U*	*P*	*ε(P)*	*Yield (%)*
Phenol–water preparation							
Fraction 1	38.5	11.5	36.5	13.5	6.8	7450	28
2	35.1	17.6	31.4	15.9	6.5	8150	37
3	32.2	21.9	28.8	17.1	6.9	8500	35
Phenol–NDS preparation							
Fraction 1	36.0	17.5	31.4	15.1			50
2	33.4	19.8	30.8	16.0			30
3	30.9	24.2	27.6	17.7			20

TABLE II

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE FRACTIONS OBTAINED BY RE-PRECIPITATION OF FRACTION 1 FROM RAT-LIVER RNA WITH 2-ETHOXYETHANOL

Fraction	*G*	*A*	*C*	*U*	*Yield (%)*
Phenol–water preparation					
1/1	42.4	9.4	34.4	12.8	57
1/2	38.4	15.4	32.6	13.7	43
Phenol–NDS preparation					
1/1	38.0	15.3	32.2	14.5	60
1/2	35.2	18.1	30.6	16.1	15
1/3	30.8	20.2	30.0	19.0	26

TABLE III

PERCENTAGE BY WEIGHT OF RESIDUAL AMINO ACIDS ATTACHED TO FRACTIONS 1, 2 AND 3 FROM RAT-LIVER RNA (PHENOL–WATER PREPARATION)

The amino acids are expressed as a percentage of the whole air-dried RNA. Valine was also present. Abbreviations: Lys, lysine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Ala, alanine; Ser, serine; Threo, threonine; Leu, leucine + isoleucine.

Fraction	*Lys*	*Arg*	*Asp*	*Glu*	*Ala*	*Ser*	*Threo*	*Leu*
1	0.62	0.31	0.30	0.44	0.29	0.25	0.26	0.24
2	0.12	0.07	0.25	0.41	0.10	0.07	0.08	
3	0.12	0.02	0.21	0.40	0.12	0.09	0.09	

TABLE IV

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE DEGRADATION PRODUCTS OBTAINED BY ETHANOL PRECIPITATION AFTER REACTION OF FRACTIONS 1/1, 2 AND 3 FROM RAT-LIVER RNA WITH RIBONUCLEASE

Abbreviations as in Table I.

Degradation product from fraction	*G*	*A*	*C*	*U*	*Recovery (%)*
1/1	54.2	15.2	21.2	9.4	55
2	51.7	16.5	21.3	10.5	55
3	45.8	22.8	21.0	10.3	45

TABLE V

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE FRACTIONS RECOVERED BY ETHANOL PRECIPITATION AFTER REACTION OF FRACTION 1/1 FROM RAT-LIVER WITH RIBONUCLEASE FOR DIFFERENT TIMES

Time of reaction with ribonuclease	*G*	*A*	*C*	*U*	*Recovery (%)*
15 min	38.5	14.0	32.0	15.6	80
30 min	38.5	14.2	32.1	15.2	64
60 min	39.5	14.1	31.0	15.2	64

Chicken liver RNA prepared by the phenol–water method could be fractionated in a manner similar to rat-liver RNA prepared by the same method.

Chicken-liver RNA prepared by the phenol–NDS method yielded a much higher proportion of fraction 1 (75 %) and this was fractionated further by the use of 2-ethoxyethanol.

RNA from regenerating rat liver produced the same proportions of fractions 1, 2 and 3 whether this RNA was prepared in the presence or absence of NDS.

The fractionation of RNA could be extended by addition of 2-ethoxyethanol to solutions of fractions 1. The basis of the separation appeared the same as before and the base compositions of the fractions obtained from fraction 1 are shown in Table II.

Amino acids

RNA prepared by the phenol–water method usually contained 1–1.5 % residual amino acids, the greatest amount of which were attached to fraction 1. The distribution of the amino acids in the 3 fractions is shown in Table III. As these were determined after 2-way paper chromatography the amounts are unlikely to be absolute, but indicate the differences between the fractions. RNA prepared by the phenol–NDS method always contained less residual amino acids (0.5 %) but a larger proportion remained attached to fraction 1 than to fractions 2 and 3.

In addition to differences in residual amino acids and base compositions the 3 fractions also differed in their solubilities in 40 % (w/v) ammonium sulphate. Fraction 1 was almost insoluble, fraction 2 was partially soluble while fraction 3 was completely soluble. The fractions also showed different C.C.D. curves.

Yeast RNA

Yeast RNA, purified as described in the experimental section, gave no precipitate upon addition of one portion of 2-butoxyethanol to a solution in 2 % potassium acetate, but a precipitate (78 %) was obtained upon adding the second portion of 2-butoxyethanol. A second precipitate (22 %) was isolated after addition of ethanol. The base compositions of the 2 fractions were nearly identical (G:A:C:U, 29:25:23:23).

Action of ribonuclease on fractions 1/1, 2 and 3 from rat liver

The base composition of the material insoluble in ethanol after treating fractions 1/1, 2 and 3 from rat liver with pancreatic ribonuclease during 2 h are shown in Table IV. These 3 materials were completely soluble in 40 % w/v ammonium sulphate and in solvent system 2 for C.C.D.

Action of ribonuclease on fraction 1/1 from rat liver at different time intervals

The base compositions of the materials precipitated by ethanol after reaction of fraction 1/1 from rat liver with pancreatic ribonuclease during 15, 30 and 60 min are shown in Table V.

Action of ribonuclease on rat liver RNA in the absence and presence of NDS

The base composition of the original RNA, and the materials recovered by ethanol precipitation after reaction with ribonuclease in the absence and presence of NDS are shown in Table VI.

TABLE VI

BASE COMPOSITIONS IN MOLAR PROPORTIONS OF THE MATERIAL RECOVERED BY ETHANOL PRECIPITATION AFTER REACTION OF RAT-LIVER RNA WITH RIBONUCLEASE IN THE ABSENCE AND PRESENCE OF NDS

Material	*G*	*A*	*C*	*U*	*Recovery (%)*
RNA	34.5	18.2	31.1	16.2	88
RNA + RNAase	40.5	15.5	29.4	14.6	64
RNA + RNAase + NDS	39.4	16.3	29.7	14.6	82

Counter current distribution

C.C.D. has only recently been applied to RNA. WARNER AND VAINBERG[13] used a mixture of 2-propanol, formamide and phosphate buffer for separating synthetic polynucleotides, and HOLLEY AND MERRILL[14] have recently applied this system to the RNA from supernatant fraction of rat-liver homogenate.

The first problem was the choice of solvents, since nucleic acids are easily precipitated from salt solutions by organic solvents. The cellosolve-salt solution used by PORTER[10] was tried and some satisfactory systems consisting of diethyl carbitol, 2-ethoxyethanol and phosphate solutions were found for commercial yeast RNA. These systems were of no use for rat-liver RNA, which was largely insoluble in the mixture used for yeast RNA. It had been found that rat-liver RNA was completely extracted into the organic phase of a system of 2-methoxyethanol and phosphate buffer and this partition has been made the basis of the isolation of RNA (and deoxyribonucleic acid) free from glycogen. When 2-butoxyethanol and phosphate buffer were used in proportions approximating to those at the plait point, all the RNA was found in the aqueous phase. From these considerations empirical mixtures have been made from 2-methoxyethanol, 2-butoxyethanol and phosphate solutions, restrictions in choice being made by the solubility of the RNA, the partition of the RNA and the volume relationship of the 2 phases. Generally an increase in the proportion of 2-methoxyethanol or of phosphate ions increased the partition in favour of the upper phase, while an increase in the proportion of 2-butoxyethanol or of water resulted in a partition in favour of the lower phase.

It has not proved possible to dissolve whole RNA directly in the solvent systems for C.C.D., but the RNA remained soluble after being dissolved in the water first and subsequently adding the phosphate solutions and then the organic solvents. HAUSSMAN AND CRAIG[15] have recently employed a similar device for the dissolution of proteins for C.C.D.

The C.C.D. curves for rat-liver RNA prepared by the phenol–water and phenol–NDS methods are shown in Fig. 1 and 2 respectively. The position of the peaks was the same in preparations by the same method but the relative heights varied from one preparation to another, especially with NDS method. The curve obtained with chicken-liver RNA made by the phenol–NDS method was similar to Fig. 2 but very much less material travelled in the organic phase.

The C.C.D. curves for fractions 1, 2 and 3 from rat-liver RNA prepared by the phenol–water method are shown in Fig. 3. The whole RNA did not show the considerable peak in tube 1 that was present in fraction 1. Fraction 3 was the only one

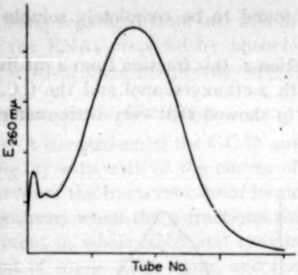

Fig. 1. C.C.D. curve for rat-liver RNA (phenol–water preparation) in solvent system 1. The maxima occur at tubes 2 and 34 after 96 transfers.

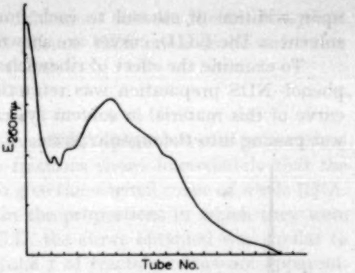

Fig. 2. C.C.D. curve for rat-liver RNA (phenol–NDS preparation) in solvent system 1. The maxima occur at tubes 1 and 26 after 96 transfers.

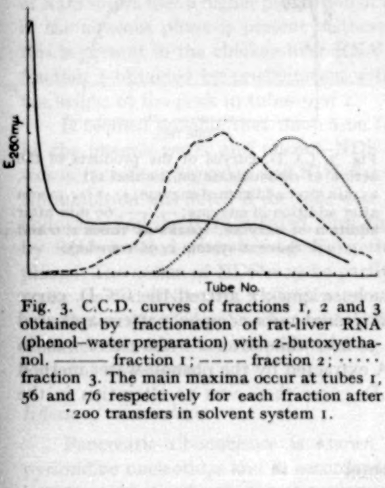

Fig. 3. C.C.D. curves of fractions 1, 2 and 3 obtained by fractionation of rat-liver RNA (phenol–water preparation) with 2-butoxyethanol. ——— fraction 1; – – – – fraction 2; · · · · fraction 3. The main maxima occur at tubes 1, 56 and 76 respectively for each fraction after 200 transfers in solvent system 1.

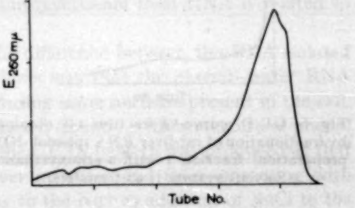

Fig. 4. C.C.D. curve of fraction 3 of rat-liver RNA (phenol–water preparation) in solvent system 2. The maximum occurs at tube 78 after 96 transfers.

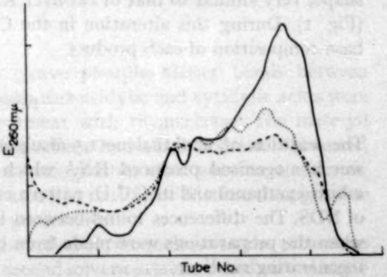

Fig. 5. C.C.D. curves of the products of the action of ribonuclease on fractions 1, 2 and 3 from rat-liver RNA (phenol–water preparation) in solvent system 2. – – – – – fraction 1 (maxima at tubes 0, 42 and 70); . . . . . . , fraction 2 (main maximum at tube 65); ——— fraction 3 (main maximum at tube 72).

completely soluble in solvent system 2 and the curve obtained is shown in Fig. 4. The peak at tube 78 was also found when yeast RNA ("purified" as described) was distributed in the same system.

When the 3 fractions from rat-liver RNA prepared by the phenol–water method were separately treated with ribonuclease, the insoluble material which separated upon addition of ethanol to each fraction was found to be completely soluble in solvent 2. The C.C.D. curves are shown in Fig. 5.

To examine the effect of ribonuclease on fraction 1, this fraction from a rat-liver phenol–NDS preparation was refractionated with 2-ethoxyethanol and the C.C.D. curve of this material in solvent system 1 (Fig. 6) showed that very little material was passing into the organic phase.

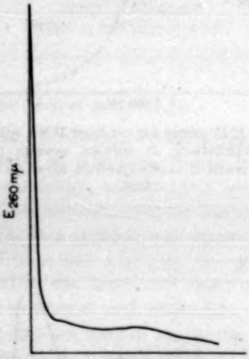

Fig. 6. C.C.D. curve of fraction 1/1 obtained by fractionation of rat-liver RNA (phenol–NDS preparation) fraction 1 with 2-ethoxyethanol. Solvent system 1, 96 transfers.

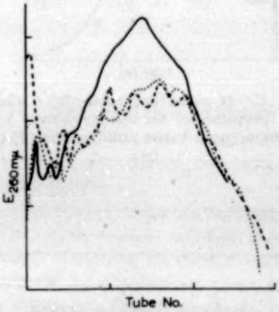

Fig. 7. C.C.D. curves of the products of the action of ribonuclease on fraction 1/1. – – – –, 15 min after addition of enzyme; · · · · · ·, 30 min after addition of enzyme; ———, 60 min after addition of enzyme; (peaks at tubes 2, 6 and 24). Solvent system 1, 96 transfers.

Treatment of this fraction with ribonuclease quickly altered the C.C.D. curve and after 15 min the high peak in tube O disappeared and a complex pattern appeared (Fig. 7). A peak at tube 24 appeared after 30 min while after 1 h the curve was, in shape, very similar to that of rat-liver RNA extracted by the phenol–water method (Fig. 1). During this alteration in the C.C.D. curve there was no alteration in the base composition of each product.

DISCUSSION

The addition of naphthalene-1,5-disulphonic acid to the water in which the tissue was homogenised produced RNA which differed in its mode of fractionation with 2-butoxyethanol and its C.C.D. pattern compared with RNA prepared in the absence of NDS. The differences found between the RNAs from rat liver were more marked when the preparations were made from chicken liver and less marked in RNA from regenerating rat-liver.

Fractionation

The basis of the fractionation of RNA by 2-ethoxy- and 2-butoxyethanol is not clear but possibly depends upon the cleavage of aggregates formed through hydrogen bonds of the A-U and G-C types as in the synthetic polynucleotides[17]. It is obvious from the base compositions of the fractions that in all cases a mixture rich in G-C is being separated from one rich in A-U. The differences between the fractionations of the RNAs prepared by phenol–water and phenol–NDS methods became more clear when these compounds were studied by C.C.D.

Counter current distribution

A comparison of the C.C.D. curves of rat-liver RNA (phenol–water preparation) (Fig. 1) with each of the curves of the three fractions shows immediately that the curves of the fractions cannot be summated to give the original curve of whole RNA. However, when the 3 fractions were mixed in the proportions in which they were present in whole RNA and examined by C.C.D. the curve obtained was similar to that of whole RNA again, and the peak in tube 1 of fraction 1 was not apparent. These curves indicate that RNA, as isolated by this method, is a complex mixture of components which interact with one another and are not easily separated by solvent system 1. Examination of the C.C.D. curves of the RNA prepared in the presence of NDS shows that a higher proportion of the material with a partition preponderantly in the aqueous phase is present in these preparations, and a greater proportion of this is present in the chicken-liver RNA than in the rat-liver RNA. The amount of fraction 1 obtained by precipitation with 2-butoxyethanol from RNA is related to the height of the peak in tubes 0 or 1.

It seemed possible that the reason for the difference between the RNA isolated by the phenol–water and phenol–NDS methods was that the phenol–water RNA was in fact more degraded and NDS was inhibiting some nuclease present in the cell. No inhibition was noted if the cells were extracted directly with a mixture of phenol and water and the RNA isolated, since the same C.C.D. pattern was produced as that by RNA isolated after homogenising the liver in water and then extracting with phenol. The action of NDS may be partly due to the Na^+ as addition of NaCl to the water yielded RNA with a slightly increased peak in tube 0.

Pancreatic ribonuclease was not inhibited by NDS (Table VI) but since 2 different ribonucleases have been reported to be present in rat liver[18] this failure to inhibit may not be relevant.

Effects of ribonuclease

Pancreatic ribonuclease is known to cleave phospho-diester bonds between pyrimidine nucleotides and in accordance with this uridylic and cytidylic acids were lost from fractions 1/1, 2 and 3 upon treatment with ribonuclease. The material insoluble in ethanol after treatment with ribonuclease had a high content of guanine, and since the ratio of guanine to adenine was greater after enzyme treatment, particularly in fractions 2 and 3, it must be assumed that more adenine than guanine is associated with the disrupted pyrimidine nucleotides.

The C.C.D. patterns of the products of the action of ribonuclease on the 3 reactions were very similar in solvent systems 1 and a second solvent system, based on 2-ethoxyethanol and ammonium sulphate solution, has also been used. Fraction 1/1 was completely insoluble in this system, while fraction 2 was only partly soluble. In contrast to this the products of the action of ribonuclease on the fractions were all completely soluble in this system. The C.C.D. curves again showed many similarities in a complex pattern which showed better resolution than solvent system 1. The effect of ribonuclease on fraction 1/1 was more marked than on fraction 2 and 3. Examination of the action of smaller amounts of ribonuclease on fraction 1/1 during shorter periods showed that while the base composition remained unaltered the C.C.D. pattern changed considerably. The peak at tube 0 decreased after 15 min, a second peak at tube 24 became evident and was more pronounced after 1 h. This peak is not in the same position as that of the C.C.D. pattern for RNA extracted with phenol–water, but then fractions 2 and 3 having partitions more in favour of the upper phase are not present. However, the position of this peak is in reasonable agreement with that found for the second peak of rat-liver RNA (phenol–NDS preparation) in solvent system 1 (Fig. 2). The curve, after 1 h, is quite similar in shape to that of whole RNA extracted by the phenol–water method and it is particularly noticeable that during this period of activity there was almost no change in the base composition.

These changes in the C.C.D. pattern of fraction 1/1 subsequent to the action of ribonuclease and culminating in a C.C.D. pattern similar to whole RNA extracted by the phenol–water method, indicate that some similar enzyme reaction may be taking place during the isolation of the RNA from the tissue. Moreover, it might be expected that the enzyme was less active, or more easily inhibited, in chicken than in rat liver, and perhaps more active in regenerating rat-liver.

DICKMAN AND RING[19] have shown that, prior to hydrolysis of phosphodiester linkages, pancreatic ribonuclease caused a non-hydrolytic alteration of the structure of yeast RNA and dilatometric experiments by VANDENDRIESSCHE[20] showed that addition of ribonuclease to RNA solutions produced a temporary increase in the volume of the solution, whereas hydrolysis of the RNA would have been expected to decrease the volume.

The changes observed in the C.C.D. pattern of fraction 1/1 after reaction with ribonuclease could be explained by an alteration of the structure of the RNA molecules or by a very limited hydrolysis which would not affect the base compositions. The evidence suggests that RNA, isolated by the phenol–water method is, to some extent, broken down more than RNA isolated by the phenol–NDS method. NDS may not be the most suitable salt for the purpose of inhibiting the enzyme since the C.C.D. curves of material extracted in this way show some variation and NDS does not inhibit pancreatic ribonuclease.

The suggestion that RNA extracted by the phenol–water method is somewhat degraded finds support in the low content of uridylic and cytidylic acids found in pancreatic RNA extracted by this method[11]. Since commercial pancreatic ribonuclease was inhibited *in vitro* by extraction with phenol some other nuclease may be responsible for the degradation. It is of interest that KEMP AND ALLEN[21] have prepared RNA from the pancreas of the dog by extraction with a hot solution of sodium dodecylsulphate and this RNA was not deficient in pyrimidines.

Very recently LASKOV, MARGOLIASH, LITTAUER AND EISENBERG[22] have also reported that RNA isolated from rat-liver by the phenol–water method was degraded. They attributed the degradation to impurities in the phenol and to the action of heavy metals, since they found that RNA with a greater sedimentation coefficient could be isolated if the phenol was distilled first and ethylenediamine tetraacetate was present during the isolation. It is possible that some of the degradation of the RNA which they observed may also be enzymic in nature if, in fact, the crude phenol contained some enzymic "activator" (*cf.* the degradation of RNA by Ce^{+++} and La^{+++} reported by BAMANN, TRAPMANN AND FISCHLER[23]).

Laskov *et al.*[21] starved their rats for 48 h before the preparation of RNA. It is of course possible that RNA after this period may not be the same as RNA in the non-fasting rat. The present preparations were made on animals which were not fasted and glycogen was removed by extraction with 2-methoxyethanol from phosphate buffer. Laskov *et al.*[22] stated that 2-methoxyethanol caused some degradation of RNA. This degradation was probably due to the peroxide content of the technical solvent since the material which represents fraction 1/1 in the present work remained in the aqueous phase and showed no signs of degradation during repeated extraction with 2-methoxy- and 2-butoxyethanol mixtures in the presence of phosphate ions. It was essential to purify the solvents as described in order to obtain consistent results with C.C.D.

ACKNOWLEDGEMENTS

The counter current distribution apparatus was purchased through funds made available by the British Empire Cancer Campaign.

I wish to thank Professor A. Haddow, F.R.S., for his interest and Miss G. E. Adams for technical assistance.

This investigation has been supported by grants to the Royal Cancer Hospital and the Chester Beatty Research Institute from the British Empire Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, United States Public Health Services.

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STUDIES ON THE NUCLEOSIDE PHOSPHOTRANSFERASE OF CARROT

III. ON THE SYNTHETIC ABILITY OF THE TRANSFER ENZYME

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(Received September 4th, 1959)

SUMMARY

The purified nucleoside phosphotransferase of carrot, which has been deprived of most of the contaminating phosphomonoesterase, is a versatile enzyme permitting the conversion to the 5′ nucleotides of unusual nucleosides, such as the ribosides of purine, 2,6-diaminopurine, and 5-bromouracil, and the glucoside or arabinoside of adenine. It is, moreover, able to use cyclic or 2′ nucleotides as phosphate acceptors, as shown by the production of adenosine 2′,5′-diphosphate and the cyclic adenosine (2′,3′), 5′-diphosphate. The latter is hydrolyzed by alkali to the 2′,5′- and 3′,5′-diphosphates of adenosine.

INTRODUCTION

The purification of the nucleoside phosphotransferase of plant tissues—an enzyme catalyzing the formation of 5′ nucleotides through the transfer of organically bound phosphoric acid to the 5′ position of deoxyribo- or ribonucleosides[1]—has recently been described in detail[2, 3]. This has made possible a brief investigation of the synthesizing potential of this enzyme. We study here the ability of several unusual nucleosides, and even of nucleotides, to act as phosphate acceptors. Some of the findings have been already mentioned in a preliminary note[4].

EXPERIMENTAL

Materials

A nucleoside phosphotransferase preparation of carrot, purified (step VI in a previous publication[2]) and deprived of phosphomonoesterase by treatment with Celite[3], was used in all experiments.

The following nucleosides were kindly given us by Dr. G. B. BROWN of the Sloan-Kettering Institute, New York: 9-β-D-ribofuranosylpurine (nebularine)[5, 6]; 9-α-L-

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arabofuranosyladenine[7]; 9-β-D-glucopyranosyladenine[8,9], and 2,6-diamino-9-β-D-ribofuranosylpurine[10]. All other nucleosides and nucleotides were commercial products.

Procedures

The previously described standard method for the assay of enzymic activity was employed, with sodium monophenylphosphate (100 μmoles/ml) as the donor and a nucleoside or nucleotide (20 μmoles/ml) as the acceptor in 0.1 *M* acetate buffer of pH 5.2 (protected with 0.001 % of ethyl mercurithiosalicylate)[2]. In addition to the procedures previously applied for the isolation and estimation of the products[2,3] a mixture of 79 vol. saturated ammonium sulfate, 19 vol. water, and 2 vol. isopropanol[11] was used for the chromatographic separation of the 2'- and 3'-adenylic acids and of the 2',5'- and 3',5'-diphosphates of adenosine.

RESULTS AND DISCUSSION

Some unusual nucleosides as acceptors

The versatility of the purified nucleoside phosphotransferase of carrot is exemplified in Table I which gives a qualitative survey of the production of the 5'-phosphates of several unusual nucleosides. Before being subjected to the action of the enzyme, the acceptors were all shown to be homogeneous chromatographically, and the appearance of a second adsorption zone served as indication of phosphate transfer. Purine riboside and adenine arabinoside were phosphorylated within 2 h of incubation; the other acceptors required longer periods.

TABLE I

PHOSPHATE TRANSFER TO SEVERAL NUCLEOSIDES*

Nucleoside as acceptor	*Relative chromatographic position of nucleotide***	*Absorbance ratios*					
		Nucleoside			*Nucleotide*		
		$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$
9-β-D-ribofuranosylpurine	0.47	0.74	0.26	0.06	0.74	0.32	0.15
9-α-L-arabofuranosyladenine	0.62	0.84	0.24	0.045	0.84	0.23	0.035
9-β-D-glucopyranosyladenine	0.52	0.85	0.19	0.017	0.86	0.15	0.025
2,6-diamino-9-β-D-ribofuranosylpurine	0.52	1.14	0.79	1.00	1.10	0.81	1.00
5-bromo-3-β-D-ribofuranosyluracil	0.47	0.50	1.83	1.45	0.47	1.91	1.41

* Incubation at 30° under standard conditions (100 μmoles phenylphosphate, 20 μmoles acceptor per ml of 0.1 *M* acetate buffer of pH 5.2). After chromatography in the isobutyric acid–ammonium isobutyrate solvent, the remaining nucleoside and the newly formed nucleotide were eluted with 0.01 *N* HCl and the eluates read in the spectrophotometer.

** With the R_F of the corresponding nucleoside taken as 1.00. Uridine 5'-phosphate had, under these conditions, a relative R_F value of 0.50 with respect to uridine.

Adenylic acids as acceptors

The nucleoside phosphotransferases of plants have been shown to transfer phosphate specifically to the 5' position of the acceptor[1,12]. The extensive purification of the transfer enzyme of carrot, resulting in the removal of most of the phosphomonoesterase activity[2,3], made possible the study of the behavior of nucleotides as phosphate acceptors. The two isomeric adenylic acids having a free 5′-hydroxyl, *i.e.*, the 2′- and the 3′-phosphates of adenosine, were investigated.

The results are summarized in Table II. 2′-adenylic acid functions as acceptor (Expt. 1), more than 10% of the nucleotide being converted to adenosine 2′,5′-diphosphate; at the same time a small amount of adenosine, produced by the dephosphorylation of the acceptor, gives rise to some 5′-adenylic acid by phosphate transfer. 3′-adenylic acid behaves differently (Expt. 2): it does not accept phosphate directly—adenosine 3′,5′-diphosphate could not be detected—but, being split more rapidly than the 2′ isomer, it is, *via* adenosine, converted to 5′-adenylic acid in a significant yield (15%). In the absence of phenylphosphate as the donor (Expt. 3), the mixture of 2′- and 3′-adenylic acids produces both 5′-adenylic acid and adenosine 2′,5′-diphosphate; the 3′ nucleotide acts as the phosphate donor and is, at the same time, the source of the adenosine that is converted to the 5′ isomer. It will be noticed that, had the specificity of the enzyme been unknown, the conversion of 3′- to 5′-adenylic acid could have been interpreted as evidence of intramolecular phosphate migration. In these experiments adenosine diphosphate was readily separable from the other products in the isobutyrate solvent system, as it moved much more slowly than the adenylic acids.

TABLE II

ADENYLIC ACIDS AS PHOSPHATE ACCEPTORS*

Expt. No.	*Initial composition of assay mixture (per ml)*	*Products (µmoles/ml) formed during incubation (h)*				
			3	*6*	*24*	*56*
	100 μg enzyme	Adenosine	0.2		0.6	1.4
1	100 μmoles phenylphosphate	Adenosine 5′-phosphate	0	0	0.4	0.4
	20 μmoles adenosine 2′-phosphate	Adenosine 2′,5′-diphosphate	0.5	1.2	2.2	2.2
	100 μg enzyme	Adenosine	1.1	1.7	3.3	6.4
2	100 μmoles phenylphosphate	Adenosine 5′-phosphate	0.3	0.5	2.3	3.0
	20 μmoles adenosine 3′-phosphate	Adenosine 2′,5′-diphosphate	0	0	0	0
	100 μg enzyme	Adenosine	0.7	1.5	3.8	10.8
3	20 μmoles adenosine 2′-phosphate	Adenosine 5′-phosphate	0.1	0.3	1.1	2.8
	20 μmoles adenosine 3′-phosphate	Adenosine 2′,5′-diphosphate	0	0.1	0.5	0.9

* The incubation was carried out at 30° and pH 5.2. At the beginning of the experiments none of the compounds listed as products was present in the assay mixtures. The products were separated on Whatman No. 1 filter paper in the isobutyric acid–ammonium isobutyrate solvent; the eluates in 0.01 *N* HCl were estimated spectrophotometrically.

Cyclic nucleotides as acceptors

When the cyclic nucleotides adenosine (2′,3′)-phosphate, uridine (2′,3′)-phosphate or cytidine (2′,3′)-phosphate were incubated with the enzyme and phenylphosphate, a new, more slowly moving product appeared on the chromatograms in the isobutyrate solvent. It had retained the spectral characteristics of the parent compound and represented the 5′-phosphate derivative of the cyclic nucleotide. The case of cyclic adenylic acid was investigated in more detail. The cyclic adenosine (2′,3′), 5′-diphosphate also offered a way to the preparation of adenosine 3′,5′-diphosphate which, as pointed out above, cannot be prepared by direct phosphate transfer to the 3′ nucleotide.

These transfer experiments with cyclic adenylic acid (barium salt) as the acceptor are shown in Table III. After incubation at 30° in 0.1 *M* acetate buffer (pH 5.2) for 24 h, portions were applied as stripes on Whatman No. 1 filter paper sheets and subjected to descending chromatography overnight in the isobutyric acid–ammonium isobutyrate solvent. The papers were dried in a vacuum over P_2O_5 and the slowest adsorption zone, which represented the diphosphate produced by the enzyme, was eluted with borate buffer of pH 7.0. Samples of the eluates served for spectroscopy, determination of inorganic phosphate, and for the determination of the ratio of organic phosphate to adenosine after hydrolysis with conc. $HClO_4$. It will be seen that phenylphosphate and 5′-adenylic acid were about equally effective as phosphate donors, whereas 5′-uridylic acid was less active.

TABLE III

CYCLIC ADENYLIC ACID AS PHOSPHATE ACCEPTOR*

Expt. No.	*Transfer enzyme µg/ml*	*Donor*	*µmoles/ml*	*Adenosine (2′,3′)-phosphate as acceptor µmoles/ml*	*Adenosine (2′,3′), 5′-diphosphate produced*				
					Yield µmole/ml	*Absorbance ratios* $\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	*Molar ratio org. P/adenosine*
1	100	Phenylphosphate	100	20	2.1	0.82	0.18	0.02	1.9
2	100	5′-Adenylic acid	20	20	2.0	0.81	0.21	0.04	2.2
3	100	5′-Uridylic acid	20	20	1.2	0.79	0.20	0.04	1.9

* Incubation at 30° and pH 5.2 for 24 h. See text for experimental details.

The cyclic diphosphate is hydrolyzed by alkali to a mixture of the two isomeric adenosine diphosphates, *viz.*, 2′,5′- and 3′,5′-diphosphate. A preparation of the cyclic compound, adenosine (2′,3′), 5′-diphosphate, which migrated as a single component in both the isobutyrate and the ammonium sulfate–isopropanol solvent systems (see below), was dissolved in water (1 mg/ml), cooled in an ice-bath, and made 0.5 *N* with respect to NaOH. After 30 min, the mixture was neutralized and subjected to paper chromatography in the $(NH_4)_2SO_4$–isopropanol solvent, with untreated cyclic adenosine diphosphate and adenosine 2′,5′-diphosphate, produced by enzymic phosphate transfer, as described before, as reference compounds. The treatment with alkali brought about the disappearance of the cyclic diphosphate; it produced two new, faster-moving compounds of which one coincided in position with authentic adenosine 2′,5′-diphosphate. Since both products, which had typical adenosine spectra, contained two phosphate groups per adenosine, the slower-moving new compound must have been adenosine 3′,5′-diphosphate. The results are summarized in Table IV.

Additional remarks

The purified nucleoside phosphotransferase of plant tissues permits, as was pointed out before[2], the preparation of many otherwise inaccessible 5′ nucleotides. This is exemplified by the description, in this paper, of the enzymic phosphorylation of several unusual nucleosides. It is also useful for the preparation of deoxyribonucleotides. The remarkable versatility of the transfer enzyme is likewise demonstrated by its ability to use nucleotides as acceptors. Derivatives that are normally difficult to obtain, such as the 2′,5′- and 3′,5′-diphosphates of adenosine—components of coenzyme II (TPN) and coenzyme A, respectively—can be prepared with relative ease.

TABLE IV

HYDROLYSIS OF CYCLIC ADENOSINE (2′,3′),5′-DIPHOSPHATE*

Compound	*Relative R_F before hydrolysis***	*Hydrolysis products*					
		*Relative R_F***	*Yield, % of starting material*	*Absorbance ratios*			*Molar ratio org. P/adenosine*
				$\frac{A_{250}}{A_{260}}$	$\frac{A_{280}}{A_{260}}$	$\frac{A_{290}}{A_{260}}$	
Adenosine (2′,3′),5′-diphosphate	0.82	—	—	***	***	***	***
Adenosine 2′,5′-diphosphate	—	1.70	34	0.85	0.20	0.02	2.1
Adenosine 3′,5′-diphosphate	—	1.30	66	0.82	0.22	0.03	1.8

* In 0.5 *N* NaOH for 30 min at 4°. See text for experimental details.

** Relative R_F values, with the R_F of 5′-adenylic acid taken as 1.0, in the ammonium sulfate–isopropanol–water solvent. Other relative R_F values, in the same solvent, were: cyclic adenosine (2′,3′)-phosphate 0.30; 3′-adenylic acid 0.50; 2′-adenylic acid 1.0. Adenosine 2′,5′-diphosphate produced, as described above, by enzymic phosphate transfer to 2′-adenylic acid, had a relative R_F of 1.67.

*** See Table III for data.

ACKNOWLEDGEMENT

This work was supported by a research grant from the National Science Foundation.

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VITAMIN K IN BACTERIA

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(Received August 19th, 1959)

SUMMARY

1. Bacterial strains of the species *Bacillus cereus, Bacillus subtilis, Proteus vulgaris, Sarcina flava, Staphylococcus aureus, Mycobacterium phlei, Pseudomonas* sp., *Azotobacter vinelandii* and a *Nocardia* species have been investigated for type and quantity of vitamin K.

2. By paper chromatography evidence has been obtained that three different types of vitamin K are synthesized by the different strains.

3. The quantity of vitamin K was estimated by biological test and by measuring the ultraviolet absorption of lipid extracts after previous purification by paper chromatography.

4. Two types of vitamin K have been isolated in crystalline form, one from *Bacillus cereus* and one from *Mycobacterium phlei*. The ultraviolet and infrared spectra of these compounds are discussed.

INTRODUCTION

The discovery of new naphthoquinones in bacteria has been reported by various authors. ISLER *et al.*[1] announced the existence of two kinds of vitamin K_2 in putrefied fishmeal, one with a C_{30}—side chain and another with a C_{35}—side chain. The identity of the isolated compounds was verified by synthesis. SNOW[2], NOLL[3] and BRODIE *et al.*[4] studied the naphthoquinones of mycobacteria and have reported chemical differences of these quinones from vitamin K_2.

In the present study different bacteria are investigated regarding the quantity and type of vitamin K which they produce.

EXPERIMENTAL

Media

The basal medium for most of the bacteria investigated had the following composition: casein-peptone-hydrolysate with 1 % of nitrogen, 400 ml; yeast autolysate with 1 % of nitrogen, 100 ml; tap water, 500 ml; pH was adjusted to 7.0. For *S. aureus* this medium was used without any additions. For the strains of *B. cereus, B. subtilis, Proteus vulgaris* and *Sarcina flava* 0.2 % of dextrose was added. For the *Pseudomonas* strain 6 and for the micrococcus strain 4 1.5 % of NaCl was added, because these organisms were isolated from marine water. The *Mycobacterium phlei* was grown on a synthetic Dorset medium: dextrose, 1 %; glycerol, 3 %; asparagine, 1.4 %; sodium citrate, 0.09 %; ferric citrate, 0.03 %; K_2HPO_4, 0.18 %; $MgSO_4 \cdot 7H_2O$, 0.15 %; pH 7.0. *Azotobacter vinelandii* was grown on a synthetic, nitrogen-free medium[5]: dextrose, 2 %; K_2HPO_4, 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.02 %; $FeSO_4 \cdot 7H_2O$, 0.005 %; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.0005 %; $CaCl_2 \cdot 6H_2O$, 0.02 %; pH 7.5. For the *Nocardia* sp. was used a potato-dextrose medium: dextrose, 1 %; peptone, 0.5 %; yeast extract, 0.1 %; K_2HPO_4, 0.2 %; KNO_3, 0.1 %; $MgSO_4 \cdot 7H_2O$, 0.2 %; NaCl, 0.1 %; $CaCO_3$, 0.2 %; $FeSO_4 \cdot 7H_2O$, trace; boiled extract of 200 g potatoes per liter.

Growth of bacteria and preparation of lipid extracts

The bacteria were grown in a liquid medium for 10–20 days. The cells were harvested by centrifugation and then dried by lyophilization. The dry culture was extracted overnight with peroxide-free ether–absolute alcohol (1:1). The extract was evaporated in vacuum, and the residue obtained was extracted with peroxide-free ether; then the ether was evaporated and this residue, which contained the vitamin K activity, was dissolved to known volume in *n*-hexane and used for paper chromatography.

Methods for separation and characterization of the K vitamins

The vitamins K were characterized by the paper chromatographic method of GREEN AND DAM[6], using Whatman No. 1 impregnated with Dow Corning Silicone 1107. The solvent for paper chromatography was: ethanol–glacial acetic acid–water (850:25:125). In a few cases *n*-propanol–glacial acetic acid–water (650:25:325) was used. As reference compounds were used: natural vitamin K_1 (Merck & Co.); natural vitamin K_2 (side chain C_{35}); synthetic vitamin K_2 (side chain C_{25} and C_{30}). These three compounds were kindly supplied by Hoffmann-La Roche and Co., Basle.

For quantitative estimation of the K vitamins the spots were eluted from the paper with *n*-hexane, and the u.v. spectra from 230 to 360 mμ were recorded on a Beckman Spectrophotometer DK 2. The *n*-hexane eluate from a blank strip of the chromatogram was used as a blank. This procedure incurred no difficulties of the kind reported by LESTER AND RAMASARMA[7] concerning paper chromatography of ubiquinones. Owing to too much absorption of the eluate, these investigators were not able to obtain u.v. spectra of the eluates of the chromatograms. The discrepancy between their results and ours may arise from the use of different Dow Corning Silicones for impregnation of the paper.

RESULTS AND DISCUSSION

Table I shows the R_F values for the reference compounds (solvent: ethanol–glacial acetic acid–water (850:25:125)) and the values of $E^{1\%}_{1\,\mathrm{cm}}$ at 248 mμ. The extinction coefficients of the three K_2 compounds are those given by ISLER *et al.*[1].

Table II shows the R_F values of the K-vitamins in the bacterial extracts and the calculated quantity of vitamin K expressed as vitamin K_2 with side chain C_{35}. For calculation the absorption peak at 248 mμ was used. The vitamin K content of some of the bacteria was tested biologically on vitamin K-deficient chicks according to the method of DAM *et al.*[8]. In this test the biological activity was compared with Menadione as reference standard. For comparison with the results of the u.v. measurement the data in Table II are presented as the effect of vitamin $K_{2(35)}$ by multiplying the data directly obtained by the ratio of the molecular weights ($K_{2(35)}$/Menadione). However, WISS *et al.*[9] have pointed out that the vitamin K activity in K-deficient chicks is dependent on the length of the side chain of the vitamin K compound; this must be taken into consideration in judging the accuracy of the figures.

TABLE I

R_F VALUES AND EXTINCTION COEFFICIENTS OF REFERENCE COMPOUNDS

Solvent for paper chromatography: ethanol–glacial acetic acid–water (850:25:125).

Compound	*R_F value*	*$E^{1\%}_{1\,cm}$ (248 mμ)*
K_1	0.75	450
$K_{2(25)}$	0.80	363[1]
$K_{2(30)}$	0.60	320[1]
$K_{2(35)}$	0.45	292[1]

From Table II it is seen that *Sarcina flava* strain 50 has two spots giving a vitamin K-spectrum: one, rather small with R_F value 0.45 like that of vitamin $K_{2(35)}$, and a second, bigger spot with R_F 0.33.

The R_F values of the compounds of *Proteus vulgaris* and *Sarcina flava* were not changed by purification of the extract by chromatography on Decalso•. The *Nocardia*

TABLE II

R_F VALUES AND QUANTITIES OF VITAMIN K IN BACTERIA

Bacterial strain	*R_F-value*	*R_F-value*	*Vitamin K*	
			(mg, calculated as mg K_1(₁₀)/g culture)	
	ethanol–glacial acetic acid–water (850:25:125)	*n-propanol–glacial acetic acid–water (650:25:325)*	*u.v. measurement*	*biological test*
B. cereus strain 35*	0.45	0.70	2.6	2.75
B. cereus strain 44*	0.46			
B. subtilis strain 13*	0.48		0.33	0.38
*Proteus vulgaris**	0.33	0.59	0.46	1.7
Sarcina flava strain 50*	0.45	0.70	0.07	
	0.33	0.58		
S. aureus 209 P (ATCC 6538P)*	0.38		little	
*Mycobact. phlei***	0.22	0.52	2.3	2.3
Pseudomonas strain 6***			nil	
Micrococcus strain 4***			nil	
Azotobacter vinelandii (Kluyver)§			nil	
Nocardia sp. strain 69§§	0.30?		0.15	

* Supplied by A. Snog-Kjær, Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen.

** Supplied by Dr. H. E. Ottosen, The State Veterinary Laboratory, Copenhagen.

*** Supplied by S. B. Halkier, Technological Laboratory, Ministry of Fisheries, Copenhagen.

§ Supplied by Dr. H. L. Jensen, State Laboratory for Soil and Crop Research, Lyngby, Denmark.

§§ Supplied by T. Vincents Nissen, Department of Technical Biochemistry, Polytechnic Institute, Copenhagen.

• "Zerolit" S/F, — 60 + 90 mesh, from United Water Softeners Ltd, London.

strain 69 always gave long spots of not well-defined R_F values, even after chromatography on Decalso.

Azotobacter vinelandii is reported to contain ubiquinone (LESTER AND RAMASARMA[7]). After chromatography we found a spectrum indicating the presence of ubiquinone and no spectrum of vitamin K.

B. cereus strain 35 and *M. phlei* are very rich in vitamin K. These strains were grown in large quantities, and their naphthoquinones were purified. After extraction of the freeze-dried culture (100 g) with acetone, the extract was dissolved in petroleum ether, and dark-coloured impurities were extracted from the solution with methanol 95 %. The petroleum ether layer was evaporated, and the residue dried over P_2O_5. It was then dissolved in petroleum ether (b.p. 60–100°) and chromatographed on Decalso. The naphthoquinone was eluted with petroleum ether, 80 %, plus benzene, 20 %. The naphthoquinones were recrystallized twice from acetone. From *B. cereus* strain 35 was obtained 80 mg of yellow crystals of vitamin $K_{2(35)}$, m.p. 54° (uncorr.). *M. phlei* gave yellow crystals at —20°, m.p. —4° (uncorr.). This oil had an u.v. spectrum similar to that of vitamin K_1 and K_2, and an $E^{1\%}_{1\,\mathrm{cm}} = 255$ at 248 mμ, indicating a molecular weight of maximally 740 assuming a molecular extinction of 20,000. The infrared spectra* of the naphthoquinones from the two bacteria were compared with those of vitamin K_1 and $K_{2(35)}$. Similarity was found between the spectrum of $K_{2(35)}$ and that of the vitamin K isolated from *B. cereus* strain 35. The IR-spectrum of the naphthoquinone of *M. phlei* was more like that of vitamin K_1 than that of vitamin $K_{2(35)}$. The main differences between the spectra of vitamin K_1 and $K_{2(35)}$ were seen in the region 650–900 cm^{-1} (Fig. 1). Vitamin $K_{2(35)}$ had four strong peaks at 687, 715, 793 and 872 cm^{-1}. Two of these, at 687 and 715 cm^{-1}, were also found in vitamin K_1 and with the same relative intensity, but instead of the two strong peaks at 793 and 872 cm^{-1} vitamin K_1 had two much weaker peaks at 785 and 890 cm^{-1}. The absorption characteristics of vitamin K_1 were found in the naphthoquinone of *M. phlei* also, but they were obscured in the preparation to some extent by an absorption at 800–850 cm^{-1} (Fig. 1). Fig. 2 shows the spectrum of vitamin K_1 and that of the *M. phlei*-naphthoquinone from 1,200 to 1,700 cm^{-1}. The quinone structure is illustrated in the spectrum by the peak at 1,660 cm^{-1}, and the benzene structure by the peak at 1595 cm^{-1}. Assuming the same molecular extinction of vitamin K_1 and of the *M. phlei*-naphthoquinone, the following calculations of the molecular weight of the *M. phlei*-naphthoquinone can be made:

(1) From the peak at 1,660 cm^{-1} a molecular weight of maximally 650.

(2) From the peak at 1,595 cm^{-1} a molecular weight of maximally 700.

Calculation from the u.v. spectrum gave a molecular weight of 740 of this compound; BRODIE *et al.*[4] reported the molecular weight of the naphthoquinone of *M. phlei* to be maximally 620.

The absorption peaks at 1,375 cm^{-1} (CH_3-groups) and at 1,440 cm^{-1} (CH_2-groups) are relatively higher in the *M. phlei*-naphthoquinone than in vitamin K_1 (Fig. 2). This may suggest that the molecule of the *M. phlei*-naphthoquinone has more CH_2- and CH_3-groups than vitamin K_1.

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* The IR-spectra (650–4,000 cm^{-1}) were recorded on a Model 21 Perkin Elmer Double-beam Spectrophotometer using the technique of KBr discs (Disc-diameter: 12.5 mm). Concentrations: approximately 1 mg naphthoquinone in 300 mg KBr; vitamin $K_{2(35)}$ from Hoffmann-La Roche: 1.08 mg; vitamin $K_{2(35)}$ from *B. cereus*: 1.08 mg; vitamin K_1 from Merck: 1.22 mg; compound from *M. phlei*: 1.04 mg.

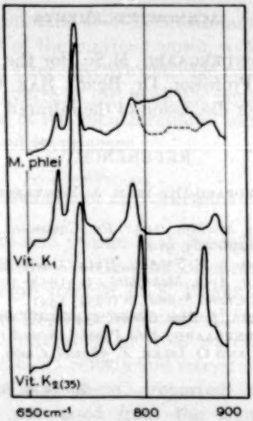

Fig. 1. Comparison of the IR-spectrum of the naphthoquinone of *M. phlei* with those of vitamins K_1 and $K_{2(35)}$. 650–900 cm^{-1}. The ordinate is optical density. The broken line represents the spectrum that would have been obtained if the substance examined had been pure vitamin K_1. Whether the difference between the solid and the broken lines is due to impurities or differences in the molecule is uncertain.

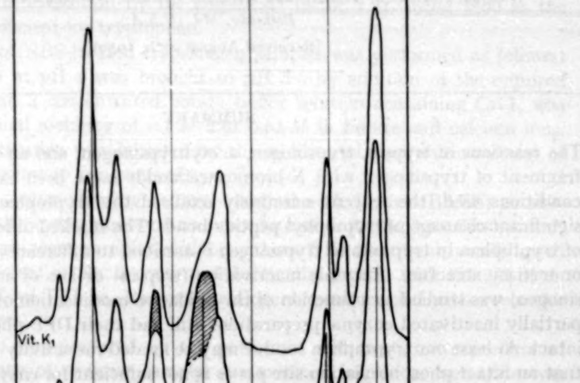

Fig. 2. Comparison of the IR-spectrum of the naphthoquinone of *M. phlei* with that of vitamin K_1. 1,200–1,700 cm^{-1}. The ordinate is optical density. The broken line in the spectrum of the naphthoquinone of *M. phlei* represents the height of the peaks if the compound were pure vitamin K_1.

ACKNOWLEDGEMENTS

We are indebted to **Ebbe Søndergaard**, M.Sc., for the biological determinations of vitamin K activity, and to Professor. Dr. **Børge Bak**, Chemical Laboratory of the University of Copenhagen, for discussion of the infrared spectra.

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THE ACTION OF N-BROMOSUCCINIMIDE ON TRYPSINOGEN AND ITS DERIVATIVES

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(Received August 27th, 1959)

SUMMARY

The reactions of trypsin, trypsinogen, acetyltrypsinogen, and an enzymically active fragment of trypsinogen with N-bromosuccinimide have been explored. Under the conditions used, the reagent selectively oxidized the tryptophan residues without significant cleavage of tryptophyl peptide bonds. The marked difference in reactivity of tryptophan in trypsin and trypsinogen is ascribed to differences in their secondary or tertiary structure. Enzymic inactivation (trypsin) or loss of activatability (trypsinogen) was studied as a function of the oxidative modification of tryptophan. Such partially inactivated enzyme preparations still had their DFP phosphorylation sites intact. At least one tryptophan residue may be needed for activity. This demonstrates that an intact phosphorylation site *per se* is not sufficient for enzymic activity.

INTRODUCTION

The selective cleavage of C-tryptophyl peptide bonds in various model peptides, the hormone glucagon, and several proteins, *e.g.*, TMV protein and serum albumins has been reported recently. Selective cleavages varying in yield from 10–40 %, have been observed using N-bromosuccinimide or N-bromoacetamide[1-3]. The action of NBS on trypsinogen, the precursor of the enzyme trypsin, was undertaken to see whether the zymogen upon activation after selective cleavage could lead to active fragments smaller than trypsin. In the course of this work the emphasis shifted from selective cleavage to selective oxidation of tryptophan residues and to the study of the properties of the modified trypsin and trypsinogen.

MATERIALS AND METHODS

Trypsinogen (1 × crystallized) and trypsin (2 × crystallized, either salt free or lyophilized) were purchased from the Worthington Biochemical Corporation, Freehold, N. J. Crystalline insulin was a gift from Dr. O. K. BEHRENS of the Eli Lilly Company, Indianapolis, Ind. N-bromosuccinimide was obtained from the Arapahoe Chemical Co., Boulder, Colorado, and recrystallized from water before use. *p*-nitrophenyl acetate was obtained from Dr. G. L. SCHMIR and recrystallized from aqueous ethanol, and ether–petroleum ether, m.p. 78–79° (reported[4] m.p. 79.5–80.0°). Benzoyl-L-arginine ethyl ester was purchased from the Mann Research Laboratories, New York.

The oxidations of trypsin and trypsinogen were carried out by slowly adding an aqueous solution of NBS (approx. 1 μmole/ml) with constant swirling, to 0.1 % solutions of the proteins in 0.1 *M* acetate buffer pH 4, until the desired amount of NBS had been added. The instantaneous oxidation of tryptophan was followed in a Cary self-recording spectrophotometer by the decrease of absorbance at 278 mμ. The extent of the oxidation of tryptophan was calculated on a molar basis by multiplying the decrease in extinction by the empirical[2] factor 1.31, using 5500 as the molar extinction coefficient for tryptophan.

The activation of NBS-treated trypsinogen samples was performed as follows: The protein solution at pH 4 was brought to pH 8.0 by addition of the required amount of alkali, and a concentrated borate buffer solution containing $CaCl_2$ was added to obtain a final molarity of 0.1 *M* and 0.02 *M* in borate and calcium ions, respectively. The activation was initiated by the addition of a catalytic amount of trypsin (1 part of enzyme to 100 parts of zymogen).

Activities were measured using benzoyl-L-arginine-ethyl ester (BAEE) as substrate according to the spectrophotometric procedure of SCHWERT AND TAKENAKA[5]. The activation of trypsinogen samples, with or without NBS treatment, was followed also by the measurement of alkali consumed during the process with the aid of an automatic titrator (Type TTT 1a, Radiometer, Copenhagen) coupled with a recorder (Ole Dich, Copenhagen).

Oxidized insulin was prepared by the performic acid oxidation procedure of SANGER[6]. The activity of enzyme samples on oxidized insulin as substrate was followed by determination of the liberated α-amino groups by reaction with ninhydrin[7].

The reaction of *p*-nitrophenylacetate (NPA) with NBS-treated trypsin samples was followed by the appearance of the *p*-nitrophenoxide absorption at 400 mμ. The reaction was started by the addition of 0.1 ml NPA solution (in ethanol) to 3.0 ml

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Abbreviations used are: NBS, N-bromosuccinimide; BAEE, benzoyl-L-arginine ethyl ester; NPA, *p*-nitrophenylacetate; DFP, diisopropylphosphofluoridate; DIP, diisopropylphosphoryl; DNP, dinitrophenyl.

of enzyme sample in 0.1 M phosphate buffer pH 7.6. The final concentration of NPA in the reaction mixture was $5 \cdot 10^{-4}$ M. Appropriate controls to correct for the spontaneous hydrolysis of NPA were included in the experiments.

The diisopropylphosphoryl (DIP) derivatives of various enzyme samples were prepared by treatment with a 50-fold excess of diisopropylphosphofluoridate (DFP) at pH 8.0 in the presence of 0.02 M calcium chloride. After 6 h no enzymic activity was left. The protein was precipitated by the addition of solid ammonium sulfate to saturation. The precipitate was collected by centrifugation, dissolved in the minimum of water and dialysed against 0.001 N hydrochloric acid for 3-4 days, with repeated changes of the dilute acid. The dialysed solutions were lyophilized.

The phosphorus content of DIP-proteins was determined by the A.O.A.C. Micro Phosphorus Method[8], as modified in the Analytical Service Laboratory of this Institute by the use of perchloric acid for rapid wet ashing. Protein concentrations were calculated from absorbance at 278 mμ of the solutions using a conversion factor of 0.6 for unoxidized samples, and suitable calculated factors in the case of NBS-treated samples. The protein concentrations so determined were confirmed by actual nitrogen determinations according to the procedure of LANNI *et al.*[9].

N-Terminal groups were analyzed by the 2,4-dinitrofluorobenzene method according to established procedures[10].

RESULTS

Oxidation of tryptophan

The addition of NBS to trypsinogen in acetate buffer at pH 4 led to a proportional and instantaneous decrease of tryptophan absorption at 278 mμ. The tryptophan content was calculated on the basis of the maximum decrease in absorbance at 278 mμ and gave 3.96 tryptophans[11] per molecule of trypsin (mol. wt. 24,000)[12]. With trypsinogen the indole absorption disappeared after the addition of 1.8 moles of NBS per mole of tryptophan. This is in keeping with the average consumption of NBS (1.5-1.6 moles) for model tryptophyl peptides. The use of the same technique on trypsin gave 3.84 tryptophans per molecule. However, in this case the reaction with NBS was not as specific as with trypsinogen, since 3.2 moles of NBS/mole of tryptophan were required to eliminate tryptophan absorption at 278 mμ. It has been shown in experiments with model compounds that tyrosine is not oxidized before tryptophan at pH 4*. Furthermore, the amino acid compositions of acid hydrolysates of trypsin and oxidized trypsin (3 % activity) were the same within experimental error (Table I). Curves showing the tryptophan destruction in the two proteins are given in Fig. 1. The products to be expected from oxidation of tryptophan in this manner are derivatives of oxindolylalanine.

Influence of NBS on the activation of trypsinogen

Trypsinogen exposed to pH 4 but not subjected to the action of NBS showed the typical activation phenomenon[14] on treatment with trypsin at pH 8. Treatment of the zymogen with NBS however, altered the activation characteristics, the nature of the change being dependent on the amount of NBS used. Fig. 2 shows that in-

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* Unpublished experiments by Dr. A. PATCHORNIK and Dr. G. L. SCHMIR.

TABLE I

AMINO ACID COMPOSITION OF TRYPSIN AND OXIDIZED TRYPSIN

Salt-free trypsin (Worthington Biochemical Corporation Lot No. 688SF) unoxidized, and oxidized as described in the text, were hydrolyzed by constant-boiling hydrochloric acid for 20 h at 105°. Except for the oxidation step, the samples were treated identically. The oxidized sample possessed 3 % of the original activity. The hydrolysates were analyzed for amino acids using the Automatic Amino Acid Analyzer (Beckman-Spinco) developed by SPACKMAN, STEIN AND MOORE[13]. 99 and 97 % of the applied nitrogen was recovered in the case of unoxidized and oxidized trypsin hydrolyzates respectively.

Amino acids	*Trypsin*		*Oxidized trypsin*	
	Number of residues per 24,000 g protein			
	Observed	*Probable*	*Observed*	*Probable*
Aspartic acid	22.0	22.0	22.2	22.0
Threonine	9.6	10.0	9.7	10.0
Serine	32.4	32.0	31.7	32.0
Glutamic acid	14.2	14.0	13.9	14.0
Proline	8.20	8.0	7.9	8.0
Glycine	25.0	25.0	25.1	25.0
Alanine	14.2	14.0	14.2	14.0
Half cystine	11.5	12.0	11.4§	12.0
Valine	11.8	12.0	11.9	12.0
Methionine	1.20	1.0	1.25	1.0
Isoleucine	12.3	12.0	12.4	12.0
Leucine	14.1	14.0	14.0	14.0
Tyrosine	9.0	9.0	8.5	8–9
Phenylalanine	3.02	3.0	2.93	3.0
Lysine	13.7	14.0	13.8	14.0
Histidine	3.10	3.0	3.10	3.0
Ammonia	29.3	29.0	29.0	29.0
Arginine	2.05	2.0	2.1	2.0

§ A very small amount of cysteic acid (0.07 mole/24,000 g protein) was observed in the chromatogram of oxidized trypsin hydrolysate.

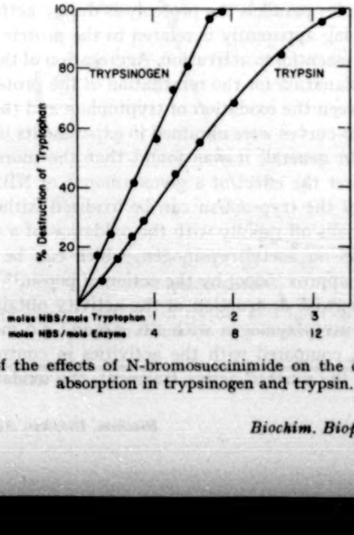

Fig. 1. Comparison of the effects of N-bromosuccinimide on the disappearance of tryptophan absorption in trypsinogen and trypsin.

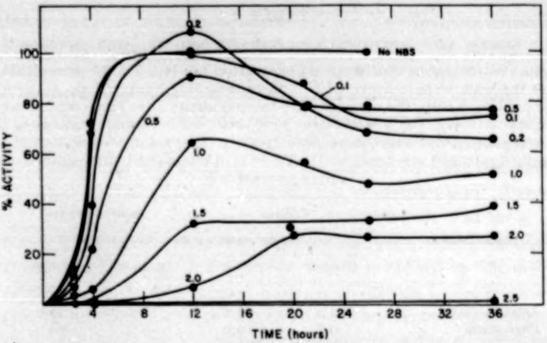

Fig. 2. Influence of various amounts of N-bromosuccinimide (moles/mole of zymogen) on the time and extent of autoactivation of trypsinogen at 25°, borate buffer pH 8 and 0.02 *M* Ca^{++}.

creasing quantities of NBS progressively lowered the extent of maximal activity. With 1.0, 1.5, 2.0 and 2.5 moles of NBS/mole of zymogen, maximum activities corresponded to 60, 45, 30 and 3 % of the control. In addition to the decrease in activity, NBS treatment also resulted in a retardation of activation, dependent on the amount of NBS used. This lag in activation was also observed on very limited oxidation (0.1 to 0.5 moles of NBS), although the same final activity as in the control was obtained. Likewise, studies on the alkali consumption during the activation of such trypsinogen samples revealed this delay. The catalytic amount of trypsin added to initiate the activation process cannot be affected by NBS, since all of the reagent is quantitatively consumed at the time of addition. Succinimide, the end product formed from NBS, was found to have no effect, either on the activation process or on the activity of trypsin. The possibility of the formation of a competitive trypsin inhibitor during the oxidation is eliminated, since oxidized trypsinogen does not interfere with the activation of intact trypsinogen. The appearance of activity in the oxidized trypsinogen samples parallels the proteolysis during activation, followed by the uptake of alkali. The lag apparently is related to the protein becoming less susceptible to the proteolysis essential to activation. Aggregation of the partially oxidized zymogen is a possible explanation for the retardation of the proteolysis.

The relationship between the oxidation of tryptophan and the maximal activity is shown in Fig. 3. The two curves were obtained in experiments in which the protein concentrations differed. In general, it was found that the more concentrated the protein solution, the greater the effect of a given amount of NBS. However, Fig. 3 shows that nearly 18 % of the tryptophan can be oxidized without much effect on the activatability, which falls off rapidly with the oxidation of a second tryptophan.

The influence of NBS on acetyltrypsinogen, which can be converted into an active fragment (mol. wt. approx. 6000) by the action of pepsin[15, 16], was also investigated. It resulted in a stepwise destruction of the activity obtained on peptic activation. Treatment of the acetylzymogen with 1.0, 2.0 and 3.0 moles of NBS led to values of 25, 13 and 3 % compared with the activities in control experiments. In this case, as well as with the purified active fragment, the oxidation of tryptophan

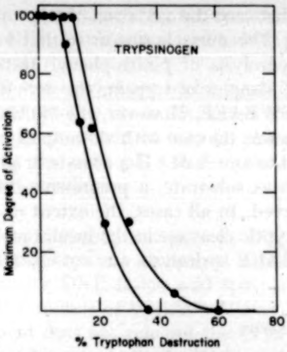

Fig. 3. Two representative runs (different volumes and concentrations) on the effects of progressive oxidation of tryptophan on the final enzymic activity of trypsinogen.

could not be followed spectrophotometrically because of precipitation of protein. The addition of 1.5 moles of NBS to the active fragment caused total and irreversible inactivation. End terminal analysis led to a single new DNP-amino acid, di-DNP-lysine in 3 % yield, probably by cleavage of the one tryptophan peptide bond present*.

Effect of NBS on trypsin

When a 0.1 % solution of trypsin in acetate buffer at pH 4 was treated with increasing quantities of NBS, no significant effect on the enzymic activity was noticed up to 3.0 moles of NBS/mole of protein. Further oxidation inactivated the enzyme. With 4.5 and 7.0 moles of NBS, 65 and 92 % of the original activity was lost. Even after treatment with 10 moles of NBS, about 2 % of residual activity was still de-

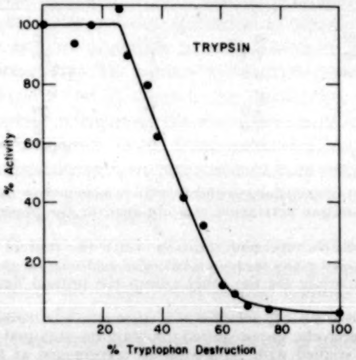

Fig. 4. The decrease of enzymic activity of trypsin as a function of the disappearance of the tryptophan chromophore caused by NBS.

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* A low yield in the cleavage reaction does not necessarily mean that the new end group is spurious. Often the yields increase when the right conditions are achieved (*cf.* ref. 3).

tectable. The relationship between the oxidation of tryptophan and the loss in tryptic activity is shown in Fig. 4. This curve is similar to that for trypsinogen (Fig. 3).

In studies on the hydrolysis of *p*-nitrophenyl acetate (NPA) by active and partially NBS-inactivated samples of trypsin, the activities towards NPA roughly paralleled activities towards BAEE. However, the "initial burst"[17] of *p*-nitrophenol was not observed, and this was the case with all samples, even the untreated control, which was merely exposed to an 0.1 *M* pH 4 acetate or phosphate buffer.

With oxidized insulin as substrate, a progressive drop in activity of oxidized trypsin samples was observed. In all cases, the extent of cleavage was restricted to the two known sites for tryptic cleavage in the insulin molecule.

The optimal pH for BAEE hydrolysis was not changed on partial oxidative inactivation of trypsin.

The incorporation of phosphorus

Enzyme preparations from oxidized trypsinogen of varying degrees of hydrolytic activity were allowed to react with DFP and the amounts of incorporated phosphorus determined. Irrespective of the activity values reached after complete activation, one mole of phosphorus per mole (24,000 g) of protein was incorporated. A partially activated sample, having approx. 3 % activity as compared with 25 % on maximum activation, contained only 0.17 mole of phosphorus. After complete activation this value increased to 1 mole (Table II).

Oxidized trypsin samples, inactivated to various degrees, were also reacted with DFP. Samples of low tryptic activity still bound one mole of phosphorus per mole

TABLE II

THE INCORPORATION OF PHOSPHORUS ON DFP TREATMENT OF SAMPLES OF TRYPSINOGEN AND TRYPSIN AFTER OXIDATION WITH NBS

No.	*Enzyme*	*Moles of NBS per mole of protein*[*]	*Percent tryptophan destruction*	*Percent activity observed*[**]	*Moles of phosphorus per mole of protein*
1	Trypsinogen	0	0	100	1.04 (1.06)[***]
2	Trypsinogen	1.5	17	50	0.98 (0.98)
3	Trypsinogen[§]	2.0	30	3–5; *25*[§§]	0.17 (0.18) (*1.07*)[§§]
4	Trypsin	0	0	100	0.90 (0.92)
5	Trypsin	3.6	36	50	1.0 (0.91)
6	Trypsin	8.0	77	3	0.61 [§§§]
7	Trypsin	6.0	62	approx. 10	0.37[†] (0.37)

[*] The molecular weight of trypsinogen and trypsin is assumed to be 24,000.

[**] In the case of trypsinogen activation was obtained in the presence of catalytic amounts of trypsin.

[***] The values in parentheses represent ratios in which the protein values were obtained by spectrophotometric methods by the measurement of absorbance at 280 mμ and the use of empirical conversion factors, while for the other values the protein figures were determined by nitrogen analyses.

[§] When a large volume of a 0.2 % solution of trypsinogen was treated with 1.5 and 2.0 moles of NBS, 24 and 48 h, respectively, were required to obtain the maximal activity (50 % and 25 %). The DFP inhibition was studied with NBS-oxidized trypsinogen at two different intervals of activation.

[§§] DFP treatment of fractions with only 3–5 % sub-maximal activity resulted in an incorporation of 0.17 mole of phosphorus, which increased to 1.07 moles at maximum activity (25 %).

[§§§] The time of reaction with DFP was 20 h.

[†] The time of reaction with DFP was 6 h.

of protein. A sample which had 10 % activity incorporated 0.37 mole of phosphorus after 6 h of reaction with DFP, but a sample with only 3 % activity incorporated 0.61 mole after 18 h (Table II).

The reaction with DFP was carried out under conditions so as to minimize the phosphorylation of the second reactive site in the protein[18]. The oxidative inactivation is not reversible at pH 8, under the condition used for phosphorylation, since suitable controls which were kept at the same pH failed to show any increase in activity towards BAEE on prolonged standing.

End group analysis

Qualitative analysis for N-terminal groups on an NBS inactivated sample of trypsin showed only one major DNP-amino acid spot, corresponding to DNP-isoleucine, from the known N-terminus of trypsin[19]. However, the failure to find any other spots in this experiment does not rule out the formation of minor amounts of new N-terminal groups due to tryptophyl peptide cleavage[1-3]; it only indicates that under the inactivation conditions extensive cleavage does not occur. N-Terminal analysis after treatment of trypsinogen with twelve moles of N-bromoacetamide in 70 % acetic acid yielded DNP-amino acid spots corresponding to DNP-phenylalanine (about 20 %) and di-DNP-lysine (about 3 %), in addition to the DNP derivative of valine, the known N-terminus of trypsinogen[19].

DISCUSSION

Treatment of trypsinogen and trypsin with NBS results in the largely selective oxidation of the tryptophan residues in these proteins, as is revealed by the spectral changes, and supported by amino acid analyses (Table I). The relative resistance of the tryptophyl residues in trypsin is probably a manifestation of overall structural differences between the zymogen and the enzyme. Nearly 18 and 25 % of the four tryptophan residues in the zymogen and the enzyme, respectively, can be destroyed without impairing the catalytic activity (potential or present). This suggests that one tryptophan residue is more accessible to oxidation than the other three, and is not essential for enzymic activity. The manner in which enzymic activity is lost during further oxidation (*cf.* Figs. 2 and 3) suggests the implication of at least one of the remaining three tryptophan residues in the maintenance of catalytic activity. The active fragment (mol. wt. approx. 6000) from acetyltrypsinogen contains but one tryptophan of the four originally present. Since oxidation of this particular tryptophan inactivates the enzyme, the part of the protein molecule containing this residue probably is essential for enzymic activity.

The residual activity is an inherent property of the oxidized proteins and is not due to intact starting material. The following observations support this view:

(a) The specific activity of various NBS treated trypsins could not be altered by fractional precipitation with ammonium sulfate. (b) Enzyme samples which had lost considerable activity towards BAEE were capable of incorporating one mole of phosphorus (from DFP) per mole of protein; this rules out unoxidized protein as a source for residual activity. Trypsin which had lost nearly 98 % of its activity towards BAEE could still incorporate 0.61 mole of phosphorus, a value which is nearly 30 times the expected uptake if the residual 2 % activity were due to intact trypsin.

(c) The DIP-derivative of oxidized trypsin (which had 50 % activity prior to treatment with DFP) was found to be homogeneous in the ultracentrifuge, and to sediment at the same rate as the DIP-derivative of unoxidized trypsin.

The oxidation of trypsin by NBS affects its activity towards BAEE, a typical substrate, without impairing its ability to react with DFP. The phosphorylation site which is currently considered to be a part of the "active site" of the enzyme molecule[20-22] apparently remains unaffected during the oxidation. This observation demonstrates the necessity of other factors besides an intact phosphorylation site for the optimal function of the enzyme. Similarly, WOOD AND BALLS[23] have shown that enzymic oxidation of a single tryptophan in chymotrypsin lowered its catalytic activity to 50 % without affecting its ability to be phosphorylated. They considered tryptophan to be an auxiliary group, necessary for full activity. The inactivation may be the result of a slight disorientation* of the active configuration of the protein molecule, brought about by the modification of one or more tryptophan residues. The actual role of tryptophan in relation to enzymic activity remains to be elucidated.

ACKNOWLEDGEMENT

We wish to express our thanks to Dr. S. BLACK for critical examination of the manuscript.

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* A small increase in levorotation accompanies the oxidative inactivation of trypsin.

ACIDIC PEPTIDE CONJUGATES IN MAMMALIAN LIVER

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(Received August 27th, 1959)

SUMMARY

Examination of the trichloroacetic acid-soluble fraction of several mammalian tissues has revealed the presence of a variety of peptides that have acidic properties and that are closely associated with material showing maximum absorbance at about 268 mμ. These conjugates contained from two to seven different amino acids. Although the peptides and the materials absorbing u.v. were closely associated during the early stages of the purification, they disassociated in the course of further fractionation. The nature of the linkage between these components remains to be established.

INTRODUCTION

Studies[1] of the kinetics of amino acid incorporation into protein, demonstrating non-uniform labeling, indicate the participation of amino acid conjugates as intermediates in protein biosynthesis, but they provide no information regarding the chemical nature or molecular size of these intermediates. Over the past 3 years we have examined a number of tissue fractions for the possible presence of amino acid conjugates and describe below the occurrence of oligo-peptides in the trichloroacetic acid-soluble fraction of the kidney of the rat and the liver of the rat and the rabbit. Because of the very low concentration of these compounds, definitive characterization has not yet been accomplished. The present communication is in the nature of a preliminary report and includes no evidence implicating these compounds in protein biosynthesis.

METHODS

Tissues were removed, rapidly minced into cold 10% trichloroacetic acid (TCA) and then homogenized either in a Potter-Elvehjem apparatus or in a Waring blendor. The protein precipitate was removed by centrifugation. After extraction of the TCA with ether, the supernatant solution was lyophilized. The dry residue was taken up in a small volume of water. This solution (pH 4–5) was placed on a column of Dowex-50-H^+ ion exchange resin and the material eluted with water was collected for further fractionation. This material was lyophilized and stored in the cold as a dry powder pending analysis.

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Acid-washed Whatman No. 3 filter paper was used for paper chromatography and electrophoresis. The following chromatographic solvent systems were employed: (a) butanol–acetic acid–water (4:2:1); (b) 70% propanol, 30% water (v/v); (c) 90% methyl cellosolve, 10% water, (v/v); (d) saturated ammonium sulfate–isopropanol–water (79:2:19)[2]. High voltage electrophoresis on paper was carried out as described by MICHL[3] with pyridine–acetic acid buffer, pH 6.0. Fractions separated on paper were eluted with distilled water and, when necessary, concentrated under reduced pressure at room temperature.

Columns (1.2 × 40 cm) of ECTEOLA-formate* were washed with water until the pH of the eluate was neutral. The sample (adjusted to pH 4.5) was applied and the column was washed with approx. 400 ml water until the absorbance of the eluate was less than 0.03 at 260 mμ. Gradient elution was begun with a constant-volume mixing chamber containing 1 l of water that was fed from a reservoir containing 0.2 M ammonium formate, pH 4.3. The flow rate was 16 to 20 ml/h. Samples of 5 ml were collected. In the experiment, results of which are summarized in Fig. 1, after 800 ml of eluate had been collected, the solution in the mixing chamber was replaced with 1 l of 0.1 M ammonium formate, pH 4.3, and the elution was continued with 0.5 M ammonium formate of the same pH in the reservoir. KORN[4] has reported the separation from tissue extracts of several unidentified compounds possessing absorption at 260 mμ using a similar system. Most of the mono-, di- and triphosphates of adenosine, guanosine, uridine and cytidine can be separated by this technique and

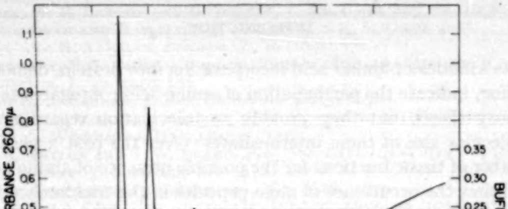

Fig. 1. Upper portion: Elution pattern of the acidic components in a trichloroacetic acid extract of rat liver chromatographed on ECTEOLA-formate and the calculated gradient of buffer molarity. *Lower portion:* Pattern of ninhydrin positive components in paper chromatograms of acid-hydrolyzed aliquots of the column eluate (butanol–acetic acid–water (4:2:1)).

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* ECTEOLA-formate was kindly provided by Dr. E. KORN, who prepared it.

at least some of the compounds separated by chromatography of liver extracts and absorbing light at 260 mμ probably represent simple nucleotides.

RESULTS

In a preliminary experiment, data from which are summarized in Table I, the kidneys from a single rat (273 mg protein) were treated as described above. The water eluate from Dowex-50-H^+ was chromatographed on paper using solvent (c). Under u.v. light several quenching bands and several fluorescent bands could be distinguished on the paper. Staining with ninhydrin revealed three bands with distinct blue or purple color which were designated K1, K2 and K3. These three bands and also a ninhydrin-negative band that was fluorescent under u.v. light (K4) were eluted and again chromatographed, this time using solvent (a). K1 and K4 each yielded only a single component. At least 3 distinct bands were identified on the chromatogram of K2. Two of these stained with ninhydrin and one did not. The latter band reacted with the molybdic acid reagent for phosphate and absorbed u.v. light. K3 was resolved into 4 components by chromatography with solvent (a). After acid hydrolysis glutamic acid, glycine, alanine and aspartic acid were identified in all 4 of the components of K3.

TABLE I

CHROMATOGRAPHIC PROPERTIES AND AMINO ACID COMPOSITION OF PEPTIDE CONJUGATES FROM RAT KIDNEY

Band number	Solvent (c)*	Solvent (a)*			Amino acids in hydrolysate**						
	R_F	R_F	Ninhydrin staining	u.v.***	Glu	Gly	Ala	Asp	Leu	Val	Thr
K-1	0.13–0.24	0.04–0.12	+	f	±	±					
K-2-1	0.24–0.30	0.03–0.06	+	f	2 +	2 +		±			
K-2-2		0.06–0.09	+	f	±						
K-2-3		0.09–0.14	—	q	3 +	2 +		1 +			
K-3-1	0.40–0.52	origin	—	f	3 +	3 +	2 +	2 +	±	±	
K-3-2		0.02–0.06	+	f	2 +	1 +	1 +	1 ±	±	±	±
K-3-3		0.06–0.11	+	q	2 +	1 +	1 +	1 +			1 +
K-3-4		0.24–0.27	—	q	3 +	3 +	2 +	2 +	1 +	1 +	1 +
K-4	0.62–0.70	0.20–0.28	—	f	2 +	2 +	1 +	1 +			

* See text.

** Glu = glutamic acid; gly = glycine; ala = alanine; asp = aspartic acid; leu = leucine; val = valine; thr = threonine.

*** f = fluorescent band; q = quenching band.

Acid hydrolysis of pure nucleotides is known to give rise to some amino acids, particularly glycine[5], as degradation products. However, the spectrum of amino acids obtained and the yield in relation to the 260 mμ absorbance show that only a small fraction of the amino acids in these hydrolysates could be derived in this way. One of the components contained in addition leucine (or isoleucine), valine and threonine, *i.e.* a total of 7 different amino acids. The hydrolysate of band K4 (after chromatography with solvent (a)) was shown to contain glutamic acid, glycine, alanine and aspartic acid. In addition to these amino acid components there were traces of several others, including some basic amino acids, in the hydrolysates of a number of the fractions.

5 g of rat liver was subjected to a similar fractionation procedure and again paper chromatography revealed a number of components containing conjugated amino acids. An extract of rat spleen yielded a pattern of components resembling that obtained from liver.

To permit more extensive fractionation and characterization, 3 kg of rabbit liver from fasted animals was treated as described above. A sample of the material eluted with water from Dowex 50-H^+, when subjected to electrophoresis for 4 h at approximately 20 V/cm, was found to contain a dozen or more u.v. quenching bands, many of which corresponded, of course, to known nucleoside phosphates present in this fraction. There were in addition, however, a number of acidic components with mobilities greater than those of any of the simple nucleoside phosphates and these components were ninhydrin-positive. One of these fractions with high mobility was eluted for further examination. Paper chromatography with solvent (a) separated 3 components of low R_F, all of which appeared on paper to absorb u.v. light and which stained with ninhydrin. Chromatography with solvent (b) separated 6 bands detected either by absorption of u.v. light or by reaction with ninhydrin. These were eluted and hydrolyzed and the hydrolysates were chromatographed in (solvent (a). Three of these hydrolysates contained a rather broad spectrum of different amino acids, 6, 8, and 9 in number, respectively.

Another portion of the original lyophilized acidic fraction was chromatographed on Dowex-1-formate, using continuously increasing concentrations of formic acid[6]. Thirteen components were separated, all of which possessed high absorbance at 260 mμ. Samples of the material contained in peaks 2, 4, 5, 6 and 8 were chromatographed on paper (solvent (a)). Each yielded one or more components which reacted with ninhydrin or which were detected under u.v. light; fraction 2 contained nine such components. When these were eluted, concentrated to a small volume and rechromatographed with the same solvent, four of the bands exhibited spots which were ninhydrin positive and which absorbed u.v. light. Hydrolysates of each of these contained one or more ninhydrin positive components. Tentative identification was made by comparison with known amino acids run on the same chromatogram. The amino acid composition of these 4 fractions is shown in Table II. Nine components were separated from Fraction 5 by paper chromatography. All of these exhibited strong u.v. absorption and all had similar absorption spectra: maximum absorption at

TABLE II

AMINO ACID COMPOSITION OF ACIDIC PEPTIDE CONJUGATES FROM RABBIT LIVER

Dowex-formate peak	*Chromatographic band (Solvent (a))*	*Amino acids in hydrolysate*
2	2–1	Histidine, glycine (lysine)
	2–2	Glycine, alanine
	2–3	Glycine, proline
	2–9	Glycine, threonine
5	5–2	Cysteine, histidine, serine, glutamic acid, threonine, (lysine)
	5–3	Cysteine, glycine, threonine
	5–4	Glycine, glutamic acid

268 mμ and minimum absorption at 235 mμ. After hydrolysis of an aliquot in 6 *N* HCl, these components were each shown to contain 2 to 7 different ninhydrin positive constituents. These were tentatively identified by comparison with known amino acids chromatographed at the same time. The amino acids found in three of the subfractions of peak 5 are indicated in Table II. Aliquots of a number of these components with both ninhydrin color and u.v. absorption were then rechromatographed using solvent (b)) but the ninhydrin positive moiety now ran with an R_F distinct from that of the u.v. absorbing moiety. Whether this dissociation represents splitting of a labile covalent linkage during processing or whether it indicates that the complex is only loosely associated is not known.

Fig. 1 presents data obtained from chromatography on ECTEOLA-formate of the Dowex-50-H^+ water eluate prepared from a single rat liver (8 g). In this experiment the preliminary steps were carried out as rapidly as possible. Lyophilization was begun less than 2 h after the liver was removed and special care was taken in adjusting the pH to hold it below pH 7.0 at all times. Immediately after drying was complete, the sample was taken up in water and placed on the ECTEOLA column. In the upper portion of Fig. 1 is plotted the absorbance at 260 mμ for each fraction along with the calculated gradient of buffer molarity. After 800 ml of eluate had been collected, the solution in the reservoir was changed to 0.5 *M* ammonium formate, pH 4.3, and the mixing chamber was emptied and refilled with 0.1 *M* ammonium formate of the same pH. At the end of the gradient elution shown in Fig. 1 the column was washed with 200 ml 0.1 *M* ammonium hydroxide and only a very small amount of material absorbing at 260 mμ was eluted, less than in any of the peaks shown in Fig. 1. A 3-ml sample from every third tube was hydrolyzed in 6 *N* HCl for 18 h in an autoclave (110°, 15 lbs.).

After removal of HCl these hydrolysates were chromatographed on paper with solvent (a). The lower portion of Fig. 1 is a diagrammatic representation of the ninhydrin staining compounds separated by this means. The R_F scale is only approximate since in some cases the solvent ran off the end of the paper and so all R_F values were adjusted relative to the movement of known amino acids used as markers on each paper. The amino acids have not been definitely identified since there was distortion in the lower portions of the chromatograms. However, the differences in the pattern of ninhydrin staining components in different fractions is readily apparent. Although only every third tube was examined in this way, the fractions of similar composition have been depicted as continuous on the assumption that the material in the intervening tubes would be qualitatively the same. Since the same pattern extended over wide stretches of the chromatogram, this seems very likely. This exploratory survey of the composition of the water eluate from Dowex-50-H^+ indicates that there are a number of different components containing amino acid in the TCA soluble fractions of rat liver. Chromatography on ECTEOLA appears to be a useful tool for their initial separation under mild conditions[4] with good resolution.

Examination of the TCA-soluble fraction of rabbit liver and rat liver has revealed the presence of a number of low molecular weight amino acid conjugates. These conjugates each contained between 2 and 7 different amino acids and the amino acids occurring in one or more components included: glutamic acid, aspartic acid, glycine, alanine, leucine (or isoleucine), valine, threonine, serine, histidine, proline, and cysteine. The acidic properties of the conjugates is not accounted for by their amino acid composition. In the early stages of fractionation these amino acid conjugates appeared to have an absorption maximum at 268 mμ, but in later chromatographic steps the ninhydrin positive components separated from the u.v. absorbing components. Since this work was initiated several laboratories[7-9] have reported the presence of nucleotide–peptide complexes in microorganisms. The results described above strongly suggest the occurrence of similar compounds in mammalian tissue. Because of the low yields obtained, however, it has not yet been possible to establish conclusively the chemical nature of the complexes.

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BIOSYNTHETIC INCORPORATION OF FLUOROPHENYLALANINE INTO CRYSTALLINE PROTEINS*

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(Received August 24th, 1959)

SUMMARY

1. [^{3}H]OFP and [^{3}H]PFP are incorporated into ovalbumin and into lysozyme, as well as into a mixed tissue protein fraction by minced hen's oviduct incubated *in vitro*. In these purified proteins, the analogue is present as an integral part of the molecule and has been found to be distributed among several peptides separated after partial degradation of the respective proteins.

2. The significance of these and of similar studies demonstrating the incorporation of amino acid analogues into otherwise apparently complete and normal proteins is discussed.

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* Preliminary reports of these studies have been published[1,2].
The abbreviations used are: PFP, *p*-fluorophenylalanine; OFP, *o*-fluorophenylalanine; TCA, trichloroacetic acid.

INTRODUCTION

The incorporation of *p*-flurophenylalanine, an amino acid not known to occur in nature, into bacterial protein has been demonstrated in several laboratories[1–5]. In these, as in most similar studies with amino acid analogues, the protein examined has been a mixture, in most cases the total TCA-precipitable protein of the bacteria or tissues studied. It is possible that the analogue contained in this material is present only in incomplete protein molecules, the biosynthesis of which was halted by the introduction of the unnatural amino acid. It is apparent from studies with bacterial mutants that some amino acid analogues can be very effectively utilized in the biosynthesis of functional proteins[6] and are therefore almost certainly incorporated into complete proteins. Isolation and characterization of proteins containing the analogue would be the necessary direct and conclusive evidence for utilization of any given amino acid analogue in protein synthesis. The studies described below were undertaken to determine whether PFP and OFP could be incorporated into purified lysozyme and ovalbumin by hen's oviduct incubated *in vitro*.

METHODS

Preparation of [^{3}H]OFP and [^{3}H]PFP

OFP and PFP were labeled with tritium by the WILZBACH[7] technique*. After removal of labile tritium, samples for use in incorporation experiments were subjected to chromatography on 1 × 20 cm columns of Dowex 50 (200–400 mesh) employing 0.2 M citrate buffer, pH 5.0, as the eluant. The two analogues behaved similarly on the column, emerging almost exactly at the effluent volume characteristic for phenylalanine. Approximately 50 % of the radioactivity placed on the column emerged with the front, but this peak of radioactivity contained no ninhydrin reactive material. The peak of ninhydrin reactive material, representing 80–90 % recovery of amino acid, coincided with a second peak of radioactivity. The material in this peak was pooled and desalted. The specific radioactivity of the chromatographically purified OFP was $2.8 \cdot 10^5$ counts/min/μmole. This specific radioactivity was unchanged by treatment with 6 N HCl under the same conditions used in later experiments to hydrolyze protein. After this acid treatment OFP was recovered from paper chromatograms developed with Solvent I (*tert*-butanol–methyl ethyl ketone–water, (2:2:1)). PFP purified by column chromatography had a specific radioactivity of $4.5 \cdot 10^5$ counts/min/μmole. PFP appears to be well separated from phenylalanine, which has a lower R_F, by chromatography on paper with Solvent I[3]. When, however, the labeled analogue was chromatographed under these conditions about 10 % of the radioactivity was found in the phenylalanine area. When the PFP band from such a chromatogram was eluted and re-chromatographed in the same solvent, radioactivity in the phenylalanine area again amounted to about 10 % of that in the PFP region. Similar data were obtained using [^{3}H]PFP which had been subjected to "acid hydrolysis". It was concluded that the appearance of radioactivity in phenylalanine area was due to "tailing" of PFP.

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* Exposure of the OFP to tritium gas was kindly carried out by Dr. K. E. WILZBACH. PFP was tritiated by the New England Nuclear Company.

Incubation of tissue and isolation of proteins

Minced hen's oviduct was incubated for 4 h at 37° in Krebs bicarbonate buffer containing [^{3}H]PFP or OFP, following which the tissue was homogenized in its incubation medium. The homogenate was centrifuged for 20 min in a Servall anglehead centrifuge at 4°. The sedimented material was washed twice with cold 5 % TCA, once with 5 % TCA at 90° for 15 min, twice with ethanol–ether (1:1), and twice with ether. This material is referred to as mixed tissue protein.

The supernatant fraction of the homogenate was made 40 % saturated with ammonium sulfate (pH 5.0) and the protein which precipitated was removed. Ovalbumin was crystallized from the remaining solution by adjusting the pH to 4.5 and adding ammonium sulfate to approx. 45 % saturation. It was recrystallized to constant specific radioactivity. Ovalbumin prepared in this way from four different incubation flasks (two containing [3-^{14}C] phenylalanine and two containing [^{3}H]OFP), when subjected to electrophoresis (sodium phosphate buffer, pH 6.8; ionic strength, 0.1) showed the two characteristic ovalbumin peaks, A_1 and A_2, but no others.

Lysozyme was then recovered from the supernatant fraction remaining after the first crystallization of ovalbumin as discussed above. Carrier lysozyme was added to the supernatant fraction, which was then dialyzed to remove ammonium sulfate and lyophilized. The lyophilized material was dissolved in water and lysozyme was crystallized from it by adjusting the pH to 9.5 and adding NaCl to a final concentration of 5 %. The crystalline lysozyme was chromatographed on IRC-50, according to the method of GONCALVES *et al.*[8]. In some experiments the lyophilized material was taken up in buffer and was chromatographed on a column of IRC-50 without further preliminary purification. The material in the lysozyme peak was dialyzed and the enzyme was crystallized. The enzymic specific activity of lysozyme isolated by these techniques was assayed with *Micrococcus lysodeikticus*[9] and was identical with that of the crystalline lysozyme used as carrier. Because of the use of carrier enzyme and because of the relatively limited incorporation rates observed in this system, only a minute fraction of the recovered lysozyme molecules contained analogue. Consequently it is not possible to determine whether or not those molecules containing analogue retained full enzymic activity.

Degradation of lysozyme and ovalbumin

Proteins were hydrolyzed for 18 h in 6 *N* hydrochloric acid in an autoclave at 15 lb. pressure. The hydrolysate, after removal of most of the acid, was placed on a column of Dowex 50-H$^+$ and the amino acids were eluted with 2 *N* ammonium hydroxide. PFP and OFP were separated from this effluent by chromatography on Whatman No. 3 paper using Solvent I.

Oxidized ovalbumin was prepared by dissolving 30 mg of 5 times crystallized ovalbumin, which had been dialyzed and lyophilized, in 0.8 ml 88 % formic acid. 0.2 ml 30 % H_2O_2 was added and the solution was allowed to stand for 1.5 h. After dilution with 5 ml of water and concentration *in vacuo* the oxidized ovalbumin was recovered by precipitation with acetone, washed with acetone and dried. It was digested with 0.5 mg chymotrypsin at pH 7.5 for 18 h at room temperature. Lysozyme, denatured by heating at 105° for 1 h, was digested similarly with chymotrypsin. The aromatic peptides from the digest of lysozyme were adsorbed on activated charcoal and eluted with a mixture of acetic acid and ethyl acetate[10].

10 mg of 5 times crystallized ovalbumin were dissolved in 0.2 ml 50% sodium bicarbonate solution and, after the addition of 0.4 ml of 10% (w/v) solution of fluorodinitrobenzene, the mixture was shaken for 2 h at room temperature. The dinitrophenyl-derivative of ovalbumin, which precipitated during the course of the reaction, was washed 3 times with water, 3 times with ethanol and 3 times with ether. After acid hydrolysis of this material and extraction with ether, the free amino acids were recovered by elution with 2 *N* NH_4OH from a column of Dowex 50.

High voltage electrophoresis on paper was employed for the separation of peptides in some experiments, using an apparatus of the type described by MICHL[11] with pyridine–acetate buffer, pH 3.7.

Assay of ^{14}*C and* ^{3}*H radioactivity*

Samples of dry protein or amino acid containing either ^{14}C or ^{3}H were dissolved in 1 *M* hyamine (*p*-di-isobutylcresoxymethoxyethyldimethylbenzyl ammonium hydroxide, Rohm and Haas Co., Philadelphia, Pennsylvania) in methanol in 5 dram glass vials, following which 15 ml of a solution of diphenyloxazole in toluene (6 g/l) was added. Radioactivity was assayed in a Tri-Carb Liquid Scintillation Spectrometer, using appropriate voltage and window settings for each isotope. Corrections were made for quenching by the use of an internal standard. Details of these procedures have been previously described[12].

RESULTS AND DISCUSSION

As shown in Tables I and II, both OFP and PFP appear to be incorporated into crystalline ovalbumin and into the mixed tissue protein fraction. With two of the OFP experiments parallel experiments were carried out using a portion of the same oviduct incubated with 5 mg [3-^{14}C]phenylalanine. The relative incorporation into ovalbumin and into the mixed tissue protein was the same for phenylalanine and for the analogue in these two experiments (Table I). These results suggest that the mechanism for ovalbumin synthesis is neither more nor less selective against the analogue than are the systems for production of the bulk of the oviduct proteins. They do not, of course, exclude the possibility that individual proteins are synthesized which contain relatively greater or lesser amounts of fluorophenylalanine than are incorporated into the ovalbumin.

In all of these studies the ovalbumin was recrystallized until the specific radioactivity was constant. For example, in the case of one sample of ovalbumin isolated

TABLE I

INCORPORATION OF ^{3}H-OFP INTO OVIDUCT PROTEINS

Expt.	*Protein fraction*	*Protein specific activity (counts/min/mg)*	
		^{3}H-OFP	*^{14}C-phenylalanine*
O-1	Ovalbumin	111	15,739
	Mixed tissue protein	104	15,512
O-2	Ovalbumin	48	3,328
	Mixed tissue protein	155	12,265

TABLE II

INCORPORATION OF ^{3}H-PFP INTO OVIDUCT PROTEINS

Expt.		*Concentration of PFP (mM)*	*Incubation time (h)*	*Protein specific activity (counts/min/mg)*
P-1	Ovalbumin	1.1	4	406
	Mixed protein			803
P-2	Ovalbumin	2.0	3	544
	Mixed protein			850

after incubation of minced oviduct with tritiated PFP specific radioactivities measured after the third, fourth and fifth recrystallizations were, respectively, 448, 399 and 413 counts/min/mg.

It has been reported that under certain conditions amino acid adenylates can be non-enzymically incorporated into protein[13,14]. This appears to be due to acylation of free amino groups on the protein by the activated amino acid. In order to rule out incorporation by such a mechanism in the present studies a sample of ovalbumin from experiment P-1 was treated with fluorodinitrobenzene. After acid hydrolysis and removal of the dinitrophenyl-amino acids essentially all of the original radioactivity was recovered in the free amino acid fraction, indicating that PFP was incorporated into the fabric of the ovalbumin molecules in a manner such that it was not free to react with fluorodinitrobenzene. In order to show that the incorporated analogue was distributed throughout the protein molecule, another sample of this ovalbumin preparation was oxidized and digested with chymotrypsin. The digest was subjected to chromatography on paper using a solvent mixture of *n*-butanol–acetic acid–water (4:2:1) and a series of arbitrary bands, numbered 1 to 10 from the origin to the solvent front, was eluted. As shown in Table III, radioactivity was present in varying amounts in several areas, indicating the presence of the analogue in a number of peptide fragments.

TABLE III

DISTRIBUTION OF ^{3}H IN PEPTIDES PRODUCED BY CHYMOTRYPTIC DIGESTION OF OVALBUMIN AND OF LYSOZYME

Ovalbumin P-1		*Lysozyme O-1*	
Chromatographic band	*counts/min ± S.D.**	*Electrophoretic band*	*counts/min ± S.D.**
1	23 ± 5.1	C	15.7 ± 1.77
2	18 ± 5.0	D	40.1 ± 1.99
3	126 ± 6.4	F	20.7 ± 1.82
4	30 ± 5.0	G	5.7 ± 1.68
5	47 ± 5.7	H	7.6 ± 1.70
6	39 ± 5.5	J	362 ± 3.83
7	63 ± 6.0		
8	34 ± 5.2		
9	70 ± 6.1		
10	44 ± 5.4		

* S.D. = Standard deviation.

In order to demonstrate that the tritium contained in ovalbumin actually represented incorporated analogue, a sample from Expt. P-1 (Table II) was hydrolyzed in 6 *N* HCl and the amino acid mixture was chromatographed on paper. The PFP band accounted for the major fraction of the radioactivity of the protein. In similar fashion samples of ovalbumin from Expts. O-1 and O-2 (Table I) were hydrolyzed and OFP was isolated by chromatography on Dowex 50. Tritium in this fraction accounted for 80% of the radioactivity found in the original ovalbumin samples. These studies indicate that very little, if any, defluoridation occurred during incubation of the labeled analogues with oviduct mince. This is consonant with the results of MUNIER AND COHEN[4] who found, using *E. coli* grown in the presence of ^{14}C-PFP, no evidence of conversion of PFP to phenylalanine. ARMSTRONG AND LEWIS[15] reported the urinary excretion of inorganic fluoride in rats fed PFP, but the fluoride may have been derived, not directly from the PFP molecule, but from some previously partially degraded product of the amino acid analogue.

Both OFP and PFP were also incorporated into lysozyme by the minced oviduct. A sample of crystalline, chromatographically pure lysozyme from P-1 was hydrolyzed with acid. That the protein radioactivity represented PFP was shown by paper chromatography of an acid hydrolysate using Solvent I. Approximately 80% of the incorporated radioactivity was found in the PFP band.

Lysozyme from Expt. O-1 was denatured and digested with chymotrypsin. From a fraction containing aromatic peptides prepared by adsorption on charcoal, eleven ninhydrin staining bands designated A through K (anode to cathode) were separated by high voltage electrophoresis on paper. In six of these peptides phenylalanine could be identified chromatographically after acid hydrolysis. Since lysozyme contains only three residues of phenylalanine per mole of protein, it is clear that, either the digestion was incomplete or that there was hydrolysis of bonds involving non-aromatic residues. The OFP-phenylalanine area was eluted and, in all 6 peptides, was found to contain radioactivity, but the amounts varied widely (Table III). Since the yields of the different peptides may also have varied widely, this observation cannot be interpreted as a result of non-uniformity of labeling.

OFP and PFP have been shown to be incorporated into proteins which are indistinguishable, by the methods employed, from normal ovalbumin or lysozyme. It has been established that tritium present in these proteins is entirely or almost entirely representative of analogue, and that PFP is incorporated into ovalbumin in such a manner that it is not free to react with fluorodinitrobenzene. Enzymic digestion of lysozyme and of ovalbumin produced several fragments from each protein that contained radioactivity. The demonstration that an amino acid analogue can be incorporated into what are otherwise apparently normal ovalbumin and lysozyme molecules indicates that the introduction of an unnatural amino acid into a protein molecule during the process of synthesis does not necessarily prevent completion of that molecule. Several years ago BLACK AND KLEIBER[16] isolated [^{14}C]norleucine from milk casein after feeding the analogue to cows. This was the first demonstration of the incorporation of an unnatural amino acid into a single purified protein, although proof of incorporation into the peptide chain was not presented. Recently [^{14}C]PFP has been found in several crystalline enzymes isolated from the muscle of a rabbit fed this amino acid[17]. A methionine requiring mutant of *B. subtilis* growing in the presence of ethionine produces enzymically active α-amylase in which one-third of the methionine is replaced by ethionine[18]. This is the most extensive replacement of an amino acid by its analogue which has been demonstrated in a single pure protein, although in the case of bacterial auxotrophic mutants growing on an analogue of the amino acid they require it may be assumed that there is essentially complete replacement of the amino acid with its analogue in the proteins which are formed[6].

From all of these observations it may be inferred that the process of protein biosynthesis is not absolutely specific. On the other hand, it seems probable that under normal environmental conditions with only normal amino acids present at normal concentrations a cell is probably capable of repeatedly reproducing a given protein without error. Although the present methods for determination of amino acid sequence in protein do not exclude the possibility that a small fraction of molecules differ in sequence from the majority, thus far errors have been observed only when the capacity of the protein synthesizing system to discriminate is severely challenged, as it is in analogue studies. For example, PFP, which has been shown by Munier and Cohen[4], to replace phenylalanine in the proteins of *E. coli*, is structurally more similar to phenylalanine than is phenylalanine to tyrosine, its closest relative in the family of naturally occurring amino acids. The atomic diameters of the hydrogen and fluoride substituents are very similar (H, 2.4 Å; F, 2.7 Å) while the hydroxyl group is considerably larger (approx. 5 Å). Because amino acid analogues can be incorporated into proteins it does not necessarily follow that protein synthesis lacks specificity under normal environmental conditions[19]. On the other hand it becomes important to recognize that the question of specificity must be posed in a quantitative rather than a qualitative sense.

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METABOLISM OF *SARCINA LUTEA*

IV. PATTERNS OF OXIDATIVE ASSIMILATION

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(Received August 7th, 1959)

SUMMARY

Freshly harvested or lyophilized cells of *Sarcina lutea* assimilate carbohydrate during the oxidation of glucose and a linear relationship exists between the glucose utilized and the carbohydrate deposited. The product appears to be a polymer composed of glucose units and it may be utilized in the absence of exogenous glucose.

Experiments with [U-^{14}C]** and [3,4-$^{14}C_2$]glucose show that 55 % of the carbon atoms of glucose are assimilated without appreciable dilution, while the lower incorporation of radioactivity from [1-^{14}C]glucose reflects the formation of unlabelled hexose from this substrate via the pentosephosphate cycle. Assimilation of pyruvate or acetate does not yield polysaccharide (or poly-β-hydroxybutyrate) and the assimilatory mechanism is shown by position-labelled substrates to depend on the tricarboxylic acid cycle. The carboxyl group of pyruvate is virtually eliminated while C-3 is incorporated to a greater extent than C-2; in the analogous case of acetate incorporation of C-2 exceeds that of C-1.

The data indicate that glucose is not assimilated via pyruvate and support the previous observation that under the conditions employed glycolysis is not reversible from pyruvate to glucose. On the other hand, polysaccharide formation from glycerol shows reversibility from triose, or an alternative route from triose to glucose.

Comparisons are made of the distribution in various fractions of the cell of the radioactivity assimilated from [U-^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]acetate.

INTRODUCTION

The aerobic micrococcus *Sarcina lutea* possesses the enzymes necessary for glycolysis yet is unable to metabolize glucose under anaerobic conditions[1]. Aerobically the glycolytic sequence accounts for some 70 % of the total glucose metabolized, the remainder being oxidized via the hexosemonophosphate oxidative (pentosephosphate) cycle[2], assimilation occurs and the carbohydrate content of the cells increases markedly[3]. The present work has been directed towards an understanding of the assimilatory processes of this organism which occur with glucose and also with pyruvate and acetate, compounds which are formed during glucose metabolism[1]. The behaviour of glycerol has also been investigated.

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** [U-^{14}C], uniformly labelled with ^{14}C.

The process of oxidative assimilation in micro-organisms, whereby the oxidation of a portion of the substrate provides energy for the assimilation of the remainder, has been reviewed by CLIFTON[4]. The majority of work on this topic has been carried out by manometric studies and gross carbon analyses of the cells, although WIAME AND DOUDOROFF[5], and more recently DOUDOROFF AND STANIER[6], have investigated the assimilation of radioactive substrates such as organic acids and glucose by *Pseudomonas saccharophila*. One problem always recognized in connexion with the oxidative assimilation of substrates containing more than two carbon atoms, is whether the substrate is assimilated intact or whether it undergoes metabolism to smaller fragments prior to the assimilatory process. In the case of lactate with *P. saccharophila*[5] and *Escherichia coli*[7] loss of the carboxyl group precedes assimilation, while the recent demonstration that glucose (and other substrates) may yield a polymer of β-hydroxybutyric acid in *Bacillus spp.*[8,9] and in *P. saccharophila* and *Rhodospirillum rubrum*[6] clearly establishes that degradation of the substrate must occur in these cases.

The results with *S. lutea* presented here demonstrate that the carbon atoms of glucose are not assimilated via pyruvate or acetate and that pyruvate is decarboxylated prior to assimilation. The assimilation of glucose has been shown to give rise to a polysaccharide composed essentially of glucose units which is utilized when the external supply of glucose is withdrawn. The assimilation of pyruvate or acetate does not yield carbohydrate but polysaccharide synthesis from glycerol indicates that the earlier stages of glycolysis are reversible.

MATERIALS AND METHODS

Organisms and media

The strain of *S. lutea* used was originally provided by Dr. E. F. GALE. It was grown in a peptone medium with forced aeration as previously described[1] where details of harvesting and lyophilization techniques are also given. In many of the experiments lyophilized cells which had had their endogenous respiration reduced to negligible levels (endogenous-diminished cells) were used[3].

Bacterial densities were determined turbidimetrically, after suitable dilution, in a Hilger Spekker absorptiometer using Chance glass filters H508 and OB2. The instrument was calibrated against bacterial dry weight.

Lactobacillus casei used for glucose degradation was a strain provided by the Bacteriology Department of this University. It was grown and harvested as described by COWGILL AND PARDEE[10].

Analytical methods

Glucose and reducing sugars were estimated by the method of NELSON[11] and fructose by the method of HEYROVSKÝ[12]. Cellular carbohydrate was determined by the anthrone technique according to TREVELYAN AND HARRISON[13]; it was found that more reproducible results were obtained if the tubes containing the anthrone reagent were allowed to stand in ice for at least 30 min before the addition of the solutions for analysis. All results are expressed in terms of glucose equivalents. It was necessary to determine the carbohydrate content of cell extracts in 75 % (v/v) ethanol and the effect of this solvent on the anthrone method was therefore investigated. With standard solutions of glucose in water and in 75 % ethanol, a higher O.D. is obtained for the ethanol standards, but the difference between the two sets of standards is directly proportional to the glucose concentration. Thus blanks for water and 75 % ethanol give the same O.D. Accordingly, glucose standards in 75 % ethanol were used for the determination of carbohydrate in 75 % ethanol extracts of cells.

Cellular carbohydrate was also determined as reducing sugar after hydrolysis of cell suspensions with 2 *N* H_2SO_4[14]. Preliminary experiments showed that, unlike *Escherichia coli*, hydrolysis was complete within 2 h and that heating for periods in excess of 5 h produced some destruction of carbohydrate, as did temperatures over 100°. The procedure adopted was: to 5 ml cell suspension (approx. 60 mg dry wt.) contained in an ampoule was added 0.4 ml concentrated H_2SO_4. The ampoule was sealed and heated in a boiling water bath for 2.5 h. After cooling the contents were transferred quantitatively with filtering to a 50 ml flask, neutralized and made to the mark. Suitable portions were taken for reducing sugar estimations.

Reducing sugar values for the carbohydrate content of *S. lutea* were always higher than those obtained by the anthrone method to the extent of approx. 5–6 % of the dry bacterial weight. However, the anthrone reagent does not react with hexosamines, constituents of the cell wall of *S. lutea* and which account for some 3 % of the dry bacterial weight[15], nor with pentose sugars to any marked extent. Agreement between anthrone determinations carried out on intact cells and on hydrolysates was always satisfactory (see Table I).

Glycerol was determined by the method of BURTON[16], and cells were examined for the presence of poly-β-hydroxybutyrate and lipid by the alkaline hypochlorite method of WILLIAMSON AND WILKINSON[17].

Radiochemicals and isotopic methods

[3,4-$^{14}C_2$]glucose was prepared from glycogen obtained from rabbit liver[18]. Liver slices from a fasted animal were incubated with 1 mC $NaH^{14}CO_3$ in the medium described by BUCHANAN, HASTINGS AND NESBETT[19]. The glycogen was isolated, purified as described by COWGILL AND PARDEE (see ref. 10, p. 158) and hydrolysed with 0.6 *N* HCl. After neutralization the glucose concentration was estimated and the specific radioactivity determined. Chromatography and autoradiography revealed a single spot in the position corresponding to a glucose marker. A portion of the material was converted to lactic acid by fermentation with a washed suspension of *Lb. casei* in phosphate buffer under an atmosphere of CO_2 and the lactate degraded to CO_2 and acetaldehyde with acidified permanganate (see ref. 10 p. 159). The CO_2 was counted as $BaCO_3$ and acetaldehyde as the 2,4-dinitrophenylhydrazone. 98.9 % of the radioactivity was located in C_3 and C_4 of the glucose molecule.

All other radiochemicals were obtained from the Radiochemical Centre, Amersham, England.

The isotopic methods employed have been described previously[2]. With the exception of $BaCO_3$ and acetaldehyde 2,4-dinitrophenylhydrazone, all specimens were counted at infinite thinness.

Chromatography and autoradiography

Sugar chromatography was carried out on Whatman No. 1 paper using the following solvents: ethanol–methanol–water (45:45:10, v/v), ethylacetate–pyridine–water (120:50:40, v/v) and phenol–NH_3–water (160 g phenol, 40 ml water, 1 ml 0.88 NH_3)[20]. Cell extracts were usually chromatographed in *sec*-butanol–formic acid–water (70:10:20, v/v)[21]. For autoradiography the chromatograms were stapled to Kodak Industrex type D film and, after a suitable period of exposure, developed with Kodak 19b developer. Sugar spots were located by dipping successively in $AgNO_3$ in acetone and KOH in ethanol.

Preparatory to chromatography samples of hydrolysates were treated with a mixture of Amberlite IRC-50 and IRA-400 (1:2.25, w/w) to remove salts and the resulting solutions were lyophilized. The dry material was taken up in a small volume of 85 % (v/v) pyridine and portions applied to chromatograms. Markers were always used and the movement relative to glucose (R_G) noted.

Fractionation procedures

The scheme described by ROBERTS *et al.*[21] was adopted in which suitable quantities of cells (usually about 10 mg wet wt.) were extracted successively with 4 ml cold 5 % (w/v) trichloroacetic acid (TCA) for 30 min, 4 ml 75 % (v/v) ethanol at 40–50° for 30 min, 2 ml ether + 2 ml 75 % ethanol at 40–50° for 15 min and 4 ml 5 % TCA at 100° for 30 min. Before plating the TCA fractions were extracted 4 times with 4 ml ether to remove TCA, the pH adjusted where necessary to approx. 7.5 and the fraction made up to its original volume. The fractionation procedure was carried out in graduated 10-ml centrifuge tubes to facilitate the measurement of volumes.

Assimilation experiments

Cell suspensions in water or buffer were incubated with the appropriate substrate in tubes (6 × 1 in) or Erlenmeyer flasks of suitable size. The former were aerated with water-saturated air *via* capillary tubes whereas the latter were shaken at 120 cyc/min in a thermostatic bath. In one experiment (Table III) the special apparatus designated B in a previous note[23] was used.

RESULTS

Assimilation of glucose

Effect of glucose oxidation on the polysaccharide content of the cells: The "structural polysaccharide" content of cells harvested from a peptone medium may vary between 9 and 12 % of the dry bacterial weight but is usually approximately 10 %, as determined by the anthrone method[3]. It undergoes no detectable diminution during the vigorous aeration of washed cell suspensions and while the endogenous metabolism is simultaneously reduced from a high to a negligible level.

When glucose is oxidized by freshly harvested or lyophilized endogenous-diminished cells a considerable deposition of cellular carbohydrate occurs which may increase the carbohydrate content to as much as 28 % of the dry bacterial weight. Less than one fiftieth of the total increase in carbohydrate content can be detected as free reducing sugar in extracts of cells and it was concluded therefore that the assimilated material represented polysaccharide[3]. Within the range of 0–40 mM glucose utilized, a linear relationship was established between the total glucose utilized and the carbohydrate assimilated by the cells; each mole of glucose oxidized yielded ~ 0.5 mole of assimilated material (calculated as glucose).

Nature of the assimilated polysaccharide: The increase in polysaccharide content which occurs when glucose is assimilated was measured both by anthrone and reducing sugar determinations and the fructose content of hydrolysates was also determined. Table I reveals that the anthrone and reducing sugar values are similar although the former gives a rather higher result. Only a very minor part of the increase (about 4.5 % of the total) can be attributed to fructose.

TABLE I

INCREASE IN CARBOHYDRATE CONTENT OF CELLS DURING ASSIMILATION OF GLUCOSE

625 mg lyophilized cells and 2 mmoles glucose in 0.134 M phosphate buffer pH 7.1, total volume 20 ml, were shaken at 37° in a 175-ml Erlenmeyer flask. Samples (5 ml) were withdrawn into 30 ml ice-cold water at the given times, centrifuged and the cell pellets washed with 30 ml water. The pellets were taken up in 5 ml water and stored in ice prior to analysis for bacterial density and carbohydrate by the anthrone method. Portions were hydrolysed with 2 N H_2SO_4 for 2.5 h and anthrone, reducing sugar and fructose determinations carried out on the neutralized hydrolysates.

Period of incubation (h)	*Carbohydrate content (% dry bacterial weight)*							
	Anthrone (intact cells)		*Anthrone (hydrolysate)*		*Reducing sugar (hydrolysate)*		*Fructose (hydrolysate)*	
	Content	*Increase*	*Content*	*Increase*	*Content*	*Increase*	*Content*	*Increase*
0	9.7	—	10.2	—	15.2	—	0.8	—
1	17.0	7.3	17.5	7.3	20.8	5.6	1.3	0.5
2	24.0	14.3	24.3	14.1	28.6	13.4	1.5	0.7

Chromatography of hydrolysates of 0-, 1- and 2-h samples of cells assimilating glucose gave a similar pattern of sugars which included glucose, fructose, ribose and glucosamine, but the intensity of the glucose spot alone showed a marked increase as the carbohydrate content of the samples increased. It would appear therefore that the carbohydrate assimilated consists essentially of a polysaccharide composed of glucose units.

Assimilation of radioactive glucose: The assimilation of radioactivity from variously labelled glucose by washed suspensions was next investigated. Fig. 1 records progress curves for the assimilation of [U-^{14}C], [1-^{14}C] and [3,4-$^{14}C_2$]glucose. It will be noticed that about 55 % of the radioactivity added as [U-^{14}C]glucose appears within the cells. When the external glucose is exhausted the value falls slightly, presumably as the result of the oxidation of some labile intermediates. With [1-^{14}C] glucose the total radioactivity assimilated is somewhat less but the oxidation of [3,4-$^{14}C_2$]glucose resembles the uniformly labelled sugar in that some 54 % of the radioactivity appears within the cells. The residual radioactivity present in the supernatants after all the glucose has been consumed is probably due to the slight accumulation of radioactive products of glucose metabolism. The apparently slower utilization of [3,4-$^{14}C_2$]glucose results from the higher concentration of this substrate supplied, namely 8.6 mM as opposed to 4 mM.

The relationship between the radioactivity assimilated and the increase in

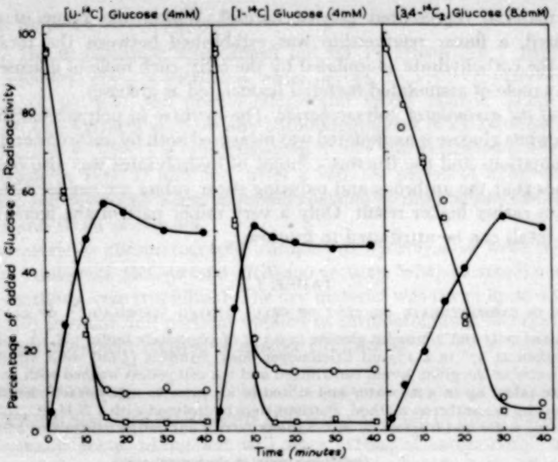

Fig. 1. Progress curves for the assimilation of variously labelled glucose by washed suspensions of *S. lutea*. ●—●, radioactivity assimilated by the cells; ○—○, radioactivity in the supernatant; □—□, glucose in the supernatant. Each system comprised 300 mg lyophilized cells, 36 μmoles glucose (77.4 μmoles in the case of [3,4-^{14}C$_2$]glucose on account of the preparation used) in a total volume of 9 ml water, gently aerated at 37°. Samples (1 ml) were withdrawn at intervals into 3 ml ice-cold water and centrifuged. The supernatants were analysed for glucose and radioactivity and the cells, after washing with 4 ml ice-cold water, were taken up to 5 ml with water and portions used for bacterial density and radioactivity assays.

polysaccharide content of the cells during the oxidation of [U-^{14}C]glucose was also studied. For this purpose it was necessary to use much higher concentrations of substrate than those employed in the assimilation experiments of Fig. 1, in order that the amount of polysaccharide deposited could be accurately measured by the methods of carbohydrate determination used (the previously established relationship between polysaccharide deposition and glucose utilization revealed that 4 m*M* glucose would give an increase in carbohydrate content of only 1.5 % of the dry bacterial weight). The data of Table II demonstrate that a good correlation exists between the two sets of values and suggest that carbohydrate accounts for all the assimilated radioactivity.

The fate of assimilated carbohydrate on prolonged incubation: The fate of the assimilated carbohydrate in the absence of an exogenous source of glucose was investigated. Cells were allowed to assimilate glucose for 2 h, washed free of glucose, suspended in phosphate buffer and then aerated at 37° for 48 h. Fig. 2 shows that after withdrawal of the external glucose the assimilated material was utilized and about 50 % disappeared within 3.5 h. Ultimately all the assimilated carbohydrate was utilized although the rate of disappearance was much less in the later stages.

This finding raised the question of whether our earlier conclusion[3] that the assimilated carbohydrate is of limited significance for endogenous respiration in *S. lutea* is valid under these particular conditions. An assessment of the problem was therefore made under conditions designed to ensure nitrogen "starvation" of the

TABLE II

CORRELATION BETWEEN RADIOACTIVITY ASSIMILATED AND INCREASE IN CARBOHYDRATE CONTENT OF CELLS DURING THE OXIDATION OF [U-^{14}C]GLUCOSE

10 ml of a suspension of lyophilized cells (320 mg dry wt.) in 0.27 *M* phosphate buffer pH 7.1 were added to 10 ml of 69.5 m*M* [U-^{14}C]glucose contained in a 175-ml Erlenmeyer flask and shaken in a thermostatic bath at 37°. The total added radioactivity was $8.88 \cdot 10^6$ counts/min. 4-ml samples were withdrawn into 40 ml ice-cold water at intervals, centrifuged, washed and then taken up with 4 ml water to give a smooth suspension. After 1 in 50 dilution 1-ml portions were used for carbohydrate determinations (anthrone) and 0.1-ml and 0.2-ml portions for assay of radioactivity. 0.5 ml was taken for fractionation and a suitable dilution was used for the determination of the bacterial density. At 120 min the residual glucose concentration was 17.47 m*M*. The carbohydrate content (anthrone) of the cells at zero time was 10.0 % of the dry bacterial weight.

	Radioactivity in cells		*Increase in cellular carbohydrate*	
Time (min)	*Counts/min/mg dry wt.*	*% of added activity assimilated*	*% dry bacterial weight*	*% of added glucose assimilated*
0.5	81	0.3	—	—
30	3600	13.1	5.8	14.8
70	6997	25.4	11.2	28.6
120	9375	34.1	13.3	34.0

cells and which might be expected to emphasize any relationship of the assimilated carbohydrate to the endogenous respiration. Endogenous diminished cells were permitted to oxidize glucose for 3 h and, after washing free of glucose, were aerated vigorously in phosphate buffer for 5 h. Samples were withdrawn at intervals and the Qo_2 value and the carbohydrate content of the cells determined. From the data of Table III it will be seen that although the Qo_2 values increase as the carbohydrate content of the cells increases, when the cells are subsequently aerated in the absence of glucose there is no simple relationship between the decrease in Qo_2 value and the

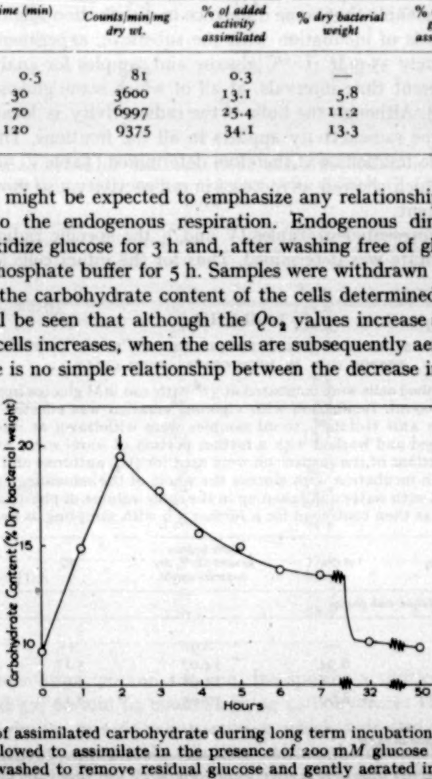

Fig. 2. The fate of assimilated carbohydrate during long term incubation of washed suspensions. The cells were allowed to assimilate in the presence of 200 m*M* glucose for 2 h, then (indicated by arrow) were washed to remove residual glucose and gently aerated in buffer at 37° for 48 h. 600 mg lyophilized cells and 2 mmoles glucose in 0.134 *M* phosphate buffer pH 7.1 (total volume 20 ml) were aerated for 2 h at 37° and then centrifuged. After washing with 0.067 *M* phosphate buffer to remove residual glucose the cells were re-suspended in 0.067 *M* phosphate buffer (20 ml) and gently aerated with water-saturated air at 37°. Samples (2 ml) were withdrawn at intervals into 2 ml ice-cold water, the cells washed and made up to 100 ml for subsequent carbohydrate determinations.

decrease in polysaccharide content of the cells. This is best appreciated by comparing the increase in Qo_2 value (ΔQo_2) effected by incubation with glucose with the increase in carbohydrate content of the cells (ΔC). The quotient $\Delta Qo_2/\Delta C$ undergoes almost a fifteen-fold change in value which clearly indicates that no set ratio exists between these two properties. Thus, after 5 h incubation in the absence of glucose, the Qo_2 has fallen by 96 % of its maximum increase whereas the carbohydrate content has decreased by only 52 % of the maximum increase attained. It would therefore appear that even under conditions of pronounced nitrogen starvation, the assimilated polysaccharide cannot be the sole nor the dominating factor in endogenous respiration.

Cell fractionation: To obtain information concerning the intracellular distribution of radioactivity, cells which had oxidized [U-^{14}C]glucose were subjected to fractionation. Since it is conceivable that some difference in distribution might occur between short and long periods of incubation with the substrate, experiments were carried out with approximately 35 m*M* [U-^{14}C]glucose and samples for analysis were withdrawn at three different time intervals, at all of which some glucose remained unconsumed (Table IV). Although the bulk of the radioactivity is located in the 75 % ethanol fraction, some radioactivity appears in all the fractions. The carbohydrate content of the soluble fractions was therefore determined (Table V) and it was found that the fractions which showed an increase in radioactivity also showed an increase in carbohydrate content.

From the data presented in Tables IV and V the specific radioactivity of the assimilated carbohydrate was determined. Thus for the intact cells and the ethanol

TABLE III

INFLUENCE OF ASSIMILATED CARBOHYDRATE ON THE LEVEL OF ENDOGENOUS RESPIRATION IN NITROGEN-STARVED CELLS

2.6 g endogenous diminished cells were incubated at 37° with 100 m*M* glucose in 0.134 *M* KH_2PO_4, pH 7.1; total volume 100 ml. Incubation with vigorous aeration was effected in the apparatus designated B by DAWES AND HOLMS[23]. 10-ml samples were withdrawn at intervals into 30 ml ice-cold water, centrifuged and washed with a further portion of 30 ml water and then taken up to 10 ml with water. Portions of the suspension were used for Qo_2, anthrone and bacterial density determinations. After 3 h incubation with glucose the whole of the remaining suspension (60 ml) was centrifuged, washed with water and taken up in the same volume of phosphate buffer (60 ml). Aeration was then continued for a further 5 h with sampling as before.

Time (h)	*Endogenous Qo_2*	*ΔQo_2*	*Carbohydrate content (C) % dry bacterial weight*	*ΔC*	*$\Delta Qo_2/\Delta C$*
	(a) Incubation with glucose				
0	4.48	—	8.90	—	—
1	11.42	6.94	14.07	5.17	1.34
2	12.66	8.18	15.98	7.08	1.16
3	13.08	8.60	17.05	8.15	1.05
	(b) Aeration in buffer				
4	9.56	5.08	15.89	6.99	0.73
5	7.28	2.80	14.46	5.56	0.50
6	6.84	2.36	13.93	5.06	0.47
7	5.49	1.01	13.43	4.53	0.22
8	4.83	0.35	12.80	3.90	0.09

TABLE IV

FRACTIONATION OF CELLS AFTER ASSIMILATION OF [U-^{14}C]GLUCOSE

Protocol as for Table II except the glucose used was 70.4 m*M*. At 120 min the residual glucose concentration was 10.45 m*M*. The carbohydrate content of the cells at zero time was 9.4% of the dry bacterial weight.

Time (min)	30		70		120	
Fraction	*Counts/min/mg dry wt. of cells*	*% of total assimilated activity*	*Counts/min/mg dry wt. of cells*	*% of total assimilated activity*	*Counts/min/mg dry wt. of cells*	*% of total assimilated activity*
Whole cells	3451	100	6517	100	9872	100
Cold TCA	644	18.7	752	11.5	1135	11.5
Ethanol	1972	57.1	3496	53.6	4655	47.1
Ethanol–ether	46	1.3	113	1.7	139	1.4
Hot TCA	340	9.9	1303	20.0	2493	25.2
Residue	341	9.9	594	9.1	538	5.4
Percentage recovery	—	96.9	—	95.9	—	90.6

TABLE V

CARBOHYDRATE CONTENT OF CELL FRACTIONS AFTER ASSIMILATION OF [U-^{14}C]GLUCOSE

Protocol as for Table II; the cells fractionated were those of Table IV. Carbohydrate determined by anthrone method.

Fraction	*Time (min)*	*Carbohydrate content µg/mg dry wt. of cells*	*Increase in carbohydrate µg/mg dry wt. of cells*
Intact cells	0.5	94.0	—
	30	141.0	47.0
	70	205.4	111.4
	120	245.0	151.0
Cold TCA	0.5	1.4	—
	30	5.3	3.9
	70	8.2	6.8
	120	13.1	11.7
Ethanol	0.5	9.7	—
	30	44.4	34.7
	70	67.9	58.2
	120	84.4	74.7
Hot TCA	0.5	47.6	—
	30	66.4	18.8
	70	77.8	30.2
	120	88.7	41.1

fraction, at times of 30, 70 and 120 min, the specific activities are respectively 73, 59 and 65, and 57, 60 and 62 counts/min/µg carbohydrate. The specific activity of the substrate glucose was 62 counts/min/µg which indicates that the substrate is assimilated without appreciable dilution. Although more divergent values are obtained for the cold and hot TCA fractions, this is scarcely surprising since both these fractions possess much smaller radioactivities and carbohydrate contents and the accuracy of the determinations must therefore be reduced.

Autoradiography of cell fractions: Autoradiograms of the ethanol and cold and hot TCA fractions each gave a single spot corresponding to an R_G value which varied on different occasions between 0.35 and 0.43 when *sec*-butanol–formic acid was the solvent. Whereas the spot from the ethanol fraction was intense those from both hot and cold TCA fractions were always faint, the cold TCA being much the weaker of the two. After hydrolysis with 1.2 *N* HCl the single spot had almost completely disappeared and a new spot, in a position corresponding to glucose, appeared. These findings, taken in conjunction with the analytical and chromatographic evidence presented earlier, indicate that the radioactive material extracted, and which is mainly located in the 75 % ethanol fraction, is a polysaccharide composed of glucose units. The chromatographic behaviour suggests that the polymer is not of high molecular weight.

Assimilation of pyruvate and acetate

Pyruvate and acetate are intermediates of glucose oxidation in *S. lutea* although pyruvate is not usually detectable unless aged cells are used or the system is inhibited by arsenite[1]. They are both oxidized without a lag period by washed cell suspensions and it was of interest, therefore, to study the pattern of assimilation of these compounds.

Analysis for polysaccharide formation: Cells were analysed for carbohydrate during the oxidation of 100 m*M* pyruvate or acetate but, unlike glucose, these substrates produced no detectable increase in the carbohydrate content of the organism as measured by the anthrone method.

Assimilation of radioactive pyruvate and acetate: Progress curves for the assimilation of [1-^{14}C], [2-^{14}C] and [3-^{14}C]pyruvate are recorded in Fig. 3 from which it will be seen that only about 7 % of the radioactivity from [1-^{14}C]pyruvate enters the cells as contrasted with some 40 and 50 % respectively from the 2- and 3-labelled substrates.

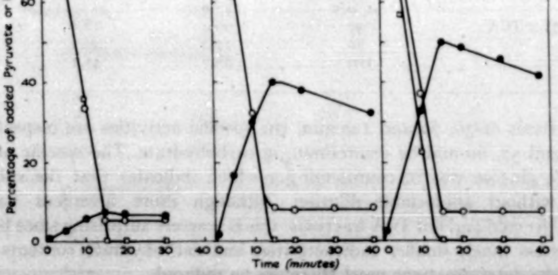

Fig. 3. Progress curves for the assimilation of variously labelled pyruvate by washed suspensions. ●—●, radioactivity assimilated by the cells; ○—○, radioactivity in the supernatant; □—□, pyruvate in the supernatant. Protocol as for Fig. 1 with pyruvate replacing the glucose.

The assimilatory patterns obtained with [1-^{14}C] and [2-^{14}C]acetate are shown in Fig. 4 and it will be noticed that more radioactivity is assimilated from methyl-labelled than from carboxyl-labelled acetate.

Cell fractionation: Cells were fractionated following the assimilation of limiting quantities (4 m*M*) of [3-^{14}C]pyruvate and [2-^{14}C]acetate, and the results are compared with the corresponding values for [U-^{14}C]glucose in Table VI. In contrast with glucose, the bulk of the radioactivity assimilated from pyruvate and acetate appears in the cold TCA fraction and not in the 75 % ethanol fraction. Comparison of the location of radioactivity in cells which have assimilated a limited amount of glucose (Table VI) with those which are assimilating in the presence of excess glucose (Table IV) reveals some differences in the distribution of radioactivity in fractions other than the 75 % ethanol extract.

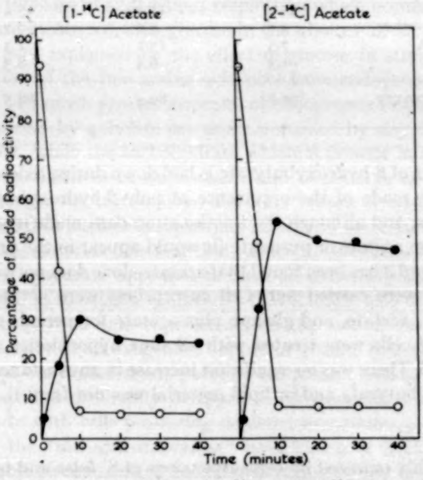

Fig. 4. Progress curves for the assimilation of [1-^{14}C]- and [2-^{14}C]acetate by washed suspensions. ●—●, radioactivity assimilated by the cells; ○—○, radioactivity in the supernatant. Protocol as for Fig. 1 with acetate replacing the glucose.

The cold TCA fraction is generally assumed to contain the more labile intermediates of the cell and the fate of the assimilated radioactivity over extended periods of incubation was therefore ascertained. Cell suspensions were allowed to assimilate limited amounts (4 m*M*) of [U-^{14}C]glucose, [3-^{14}C]pyruvate and [2-^{14}C]acetate and these substrates were exhausted during the initial 30 min. The suspensions were incubated for periods of up to 7 h and samples were withdrawn at intervals for assay and fractionation. In each case the radioactivity in the cold TCA fraction showed a marked disappearance, but only cells which had assimilated glucose displayed an appreciable loss from the 75 % ethanol fraction.

Although no attempt has been made to identify the material present in the cold TCA fractions of cells which have assimilated pyruvate or acetate, autoradiographs indicate that acetate itself is not accumulated by the cells.

Fairly recent work by various authors has demonstrated that in certain micro-

TABLE VI

LOCATION OF RADIOACTIVITY IN VARIOUS CELL FRACTIONS FOLLOWING THE ASSIMILATION OF RADIOACTIVE GLUCOSE, PYRUVATE AND ACETATE

100 mg lyophilized cells and 18 µmoles of substrate in a total volume of 3.5 ml were aerated for 25 min and then 2-ml samples withdrawn from each into 2 ml ice-cold water and centrifuged. The cells were washed twice and a further portion was diluted 10-fold for bacterial density measurement. The radioactivities present in the cells taken for fractionation were respectively (counts/min/mg dry wt.): [U-^{14}C]glucose, 3104; [3-^{14}C]pyruvate, 796; [2-^{14}C]acetate, 7403.

Fraction	*Percentage of total radioactivity*		
	[U-^{14}C]glucose	*[3-^{14}C]pyruvate*	*[2-^{14}C]acetate*
Whole cells	100	100	100
Cold TCA	27.2	58.8	62.6
Ethanol	53.0	11.2	15.4
Ethanol–ether	2.0	0.0	0.9
Hot TCA	7.3	13.1	1.3
Residue	1.3	6.4	8.3
Total recovery	90.8	89.5	88.5

organisms a polymer of β-hydroxybutyrate is laid down during oxidative assimilation. No report has been made of the occurrence of poly-β-hydroxybutyrate in *S. lutea* or related organisms, and although the fractionation data made it very unlikely that any was formed from acetate or pyruvate (it would appear in the protein residue due to its insolubility[6]), and it has been found that acetate alone does not yield the polymer[8], a few experiments were carried out. Cell suspensions were aerated with 100 m*M* solutions of glucose, acetate, and glucose plus acetate for periods of 6 h. At hourly intervals samples of cells were treated with alkaline hypochlorite and the resulting turbidities measured. There was no significant increase in any instance, thus indicating that poly-β-hydroxybutyrate and/or lipid material was not formed.

Assimilation of glycerol

Glycerol is readily oxidized by cell suspensions of *S. lutea* and in view of its close relationship to the triosephosphate stage of glycolysis, oxidative assimilation of this substrate was studied.

500 mg of lyophilized cells were incubated with 4 mmoles of glycerol and 0.134 m*M* KH_2PO_4 buffer, pH 7.1, in a total volume of 20 ml. The cellular carbohydrate content and glycerol utilization were determined at 30-min intervals over a period of 2 h. The carbohydrate content increased from 9.6 to 15.8 % of the dry bacterial weight while 738 µmoles of glycerol were consumed. From the data obtained in this and similar experiments it was calculated that each mole of glycerol utilized gives rise to 0.17 mole of assimilated material calculated as glucose.

DISCUSSION

The oxidation of glucose by *S. lutea* is accompanied by an increase in the carbohydrate content of the cells which is directly proportional to the quantity of glucose oxidized. When radioactive glucose is the substrate the radioactivity assimilated is accounted for by the glucose appearing as polysaccharide within the cells and the specific activity of this material approximates to that of the glucose substrate. Additionally chromatography of cell hydrolysates has shown glucose to be the only sugar to display a massive increase in concentration during assimilation, although the sensitive HEYROVSKÝ method for fructose did enable a slight increase in the content of this sugar to be detected. Finally, extracts of cells which have assimilated radioactive glucose contain a single radioactive component which on hydrolysis gives rise to glucose. These data together are taken to indicate that during the oxidation of glucose by non-proliferating suspensions glucose is assimilated and a polysaccharide composed essentially of glucose units is deposited in the cells.

Our earlier work[3] demonstrated that assimilated carbohydrate is unable to sustain the level of endogenous respiration characteristic of freshly-harvested cells. Furthermore, the increase in endogenous respiration which occurs when endogenous-diminished cells are incubated with glucose in the absence of an exogenous nitrogen source can be largely explained by the effect of glucose in stimulating an increase in the concentration of the free amino acid pool from endogenous peptide or polypeptide material. The much greater increases of endogenous respiration attendant on incubation with casein hydrolysate are unaccompanied by any deposition of carbohydrate in the cells, while the carbohydrate which is present in cells harvested from peptone media was shown to be quite stable and to undergo no change during the diminution of endogenous metabolism. For this reason it was assumed that the carbohydrate content of such cells represents structural material and is not a reserve of energy-producing material. The fact that, under the conditions we had been using, the assimilated carbohydrate appeared to have an extremely limited role as an endogenous reserve led us to suggest tentatively that it might also serve some structural purpose, although at that time experiments on the fate of the assimilated carbohydrate in the absence of an exogenous supply of glucose had not been carried out. This earlier concept was rendered untenable by our present finding that the assimilated material is utilized and ultimately disappears; consequently a reassessment of the problem was made with cells possessing depleted free amino acid pools. Conditions which resembled the "nitrogen-starvation" used by many workers seemed to offer the most favourable circumstances for demonstration of the role of the assimilated carbohydrate as an endogenous reserve. However, the endogenous respiration bore no simple relationship to the assimilated polysaccharide and while undoubtedly it must be making some contribution to the endogenous metabolism, it is quite clear that it is not the sole substrate utilized.

The patterns of assimilation recorded with position-labelled glucose are of considerable interest. The fact that less radioactivity is assimilated from [1-^{14}C]- than from [U-^{14}C]- or [3,4-$^{14}C_2$]glucose is a reflexion of the pathways of glucose metabolism which operate in *S. lutea*. We have already demonstrated that the pentosephosphate cycle accounts for some 30 % of the total glucose oxidized and a feature of its operation is the preferential release of carbon atom 1 of the glucose molecule. The effect of recycling [1-^{14}C]glucose by this pathway would be to produce a pool of hexosemonophosphate with lowered specific radioactivity, so that, even if the deposited polysaccharide were synthesized directly from a portion of the phosphorylated substrate, some lowering of the radioactivity of the resulting polysaccharide would be expected due to dilution with pool hexosemonophosphate.

Evidence concerning the route of synthesis of the assimilated material was also obtained. It has always been recognised by workers in this field that two possible mechanisms may be envisaged, namely (a) the assimilation of substrate molecules and their incorporation intact in the resulting cellular material and (b) the breakdown of the substrate to smaller fragments which are subsequently utilized for synthesis of the assimilated substance. Various studies [5-7, 22] have suggested that one route of glucose assimilation is *via* pyruvate which is then decarboxylated with assimilation of the two remaining carbon atoms.

That assimilation of glucose as two-carbon fragments is not the only mechanism encountered in bacteria is demonstrated by our own findings with *S. lutea*. We have also found that the carboxyl group of pyruvate is largely eliminated prior to assimilation, which indicates that pyruvate is assimilated as a two-carbon fragment such as acetate. The small amount of radioactivity (7 %) which is assimilated from [1-^{14}C]-pyruvate may well arise by CO_2-fixation reactions rather than by a small assimilation of the carboxyl group, and it is significant that we have previously demonstrated the occurrence of CO_2 fixation during glucose metabolism by *S. lutea*[2]. The metabolism of [3,4-$^{14}C_2$]glucose either by the EMBDEN-MEYERHOF or the pentosephosphate pathways gives rise to pyruvate labelled in the carboxyl group, but whereas 54 % of the activity added as the former substrate is assimilated only 7 % of the latter is found within the cells. In addition, all the activity assimilated from [U-^{14}C]glucose is accounted for by the increase in cellular polysaccharide and no other product, such as poly-β-hydroxybutyrate, for which glucose breakdown prior to assimilation would be mandatory, can be detected. Consequently the carbon atoms of glucose cannot be assimilated *via* pyruvate and the most likely explanation appears to be that a portion of the glucose substrate is assimilated as such while the remainder is being oxidized to provide energy for the assimilatory process.

The results obtained with radioactive pyruvate and acetate in the present work are in accordance with earlier conclusions[1], drawn on the basis of intact cell and cell-extract experiments, that the tricarboxylic acid cycle operates for terminal respiration in *S. lutea*. Thus more radioactivity is assimilated from methyl-labelled than from carbonyl-labelled pyruvate on the one hand, and more from methyl-labelled than from carboxyl-labelled acetate on the other. These findings are consistent with a mechanism for the preferential elimination of the carboxyl group of acetate.

The reactions of glycolysis do not appear to be reversible under the conditions we have used since no detectable carbohydrate is formed from either pyruvate or acetate. This conclusion was also reached in a previous study of the endogenous metabolism of the organism where it was shown that the assimilation of amino acids resulted in a marked stimulation of endogenous metabolism but no concomitant carbohydrate formation occurred[3]. The polysaccharide synthesis found with glycerol indicates that the block in the reversed sequence of glycolysis occurs between pyruvate and triosephosphate or, alternatively, that glucose is synthesized from glycerol by another route.

ACKNOWLEDGEMENTS

It is a pleasure to acknowledge the expert technical assistance rendered throughout by Mr J. SMILLIE, and Mr W. BURNS. Thanks are due additionally to Mr J. P. ARBUTHNOTT, who assisted in some experiments. We are also greatly indebted to the Medical Research Council for a grant-in-aid.

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Biochim. Biophys. Acta, 40 (1960) 237–251

ON THE EMISSION OF TRYPTOPHAN

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(Received September 24th, 1959)

SUMMARY

Tryptophan possesses two sets of emissions, each one of which has one short-lived emission and one long-lived emission. The excitation by π–π absorption at 280 mμ gives u.v. fluorescence and blue phosphorescence under certain conditions, *e.g.* frozen in an aqueous solution containing glucose or methanol. Both emissions are sensitive to the presence of added substances: in a frozen solution without addition, the blue phosphorescence disappears, while with the decrease in the fluorescence there is a concomitant increase in the phosphorescence, resulting from energy transfer between triplets, in the presence of acetone. The excitation by a near-u.v. absorption at 350 mμ produces blue short-lived emission and green long-lived emission, which are quenched by the addition of strong acid or the formation of charge-transfer complex with dinitrophenol, etc.

INTRODUCTION

Studies of this laboratory have indicated that tryptophan plays a special role in biological energy transmission. For this reason it seems desirable to have additional details concerning the optical properties of this amino acid. Tryptophan and proteins containing tryptophan are known to show two kinds of blue emission[1,3]. One is a long-lived emission (phosphorescence) from a triplet state on excitation at about 280 mμ. This emission is accompanied by an u.v. fluorescence from an excited singlet state. The other blue emission is a short-lived one from an unknown state of the molecule when tryptophan is excited at about 350 mμ.

The further attempt presented here to elucidate the nature of the short-lived emission has revealed that tryptophan can also emit a green long-lived emission which seems to be related electronically to the blue short-lived emission, and that the couple, the blue short-lived emission and the green long-lived emission, is subject to changes different from the other couple, the u.v. fluorescence and the blue phosphorescence, in the presence of foreign substances. This report will also present evidence that nitro-compounds, such as 2,4-dinitrophenol and trinitrobenzene, which can quench the tryptophan emission, complex with tryptophan.

EXPERIMENTAL

DL-tryptophan (Eastman Organic Chemicals) was recrystallized three times from hot methanol–water (70:30).

Emission spectra at liquid-nitrogen temperatures were measured by means of the Beckman DK-1 spectrophotometer with an unsilvered Dewar flask containing liquid nitrogen in the lamp-housing compartment[2]. The exciting light was isolated from a Hanovia high-pressure xenon arc (Model 10-C-1) using a Leiss fused-quartz monochromator.

Absorption spectra were studied with the Beckman DK-1 recording spectrophotometer.

RESULTS

The results of tryptophan emission are summarised in Table I with those of indole and protein emission for comparison.

In a frozen aqueous solution, tryptophan excited by 350 mμ was found to emit a weak green long-lived emission at liquid-nitrogen temperature, as well as the blue short-lived emission at about 450 mμ (Fig. 1). Since protein emitted more strongly, the use of a narrow slit enabled two peaks to be shown at 440 mμ and at about 500 mμ (Fig. 2). This indicates that there are two different states excited by near-u.v. light, which give rise to two kinds of emission: blue short-lived emission and green long-lived emission.

The previous spectral study of a concentrated protein solution suggested the possible existence of a long-wave-length shoulder on the 280 mμ peak[2]. Investigation of the action spectrum for the short-lived blue emission of tryptophan indicated that the emission was excited in the vicinity of 360 mμ[3]. In the present study, absorption spectra in the near u.v. region were measured for both tryptophan and

TABLE I

EMISSION OF TRYPTOPHAN, INDOLE AND PROTEIN AT 77° K

Exciting light	*280 mμ*				*350 mμ*	
Emission	*Short-lived*		*Long-lived*		*Short-lived*	*Long-lived*
Tryptophan	325 mμ	345	410 435 435	~500	450	~500
Water	—	+	—	+	+	+
Glucose	++	—	++	—	+	+
Methanol	++	—	++	—	+	+
Acid	+	—	+	—	—	—
Acetone	—	—	+	+	+	+
Cysteine	—	—	—	+	+	+
Histidine	+	—	+	—	+	+
Dinitrophenol Riboflavin Folic acid	—	—	—	—	—	—
Indole	310 320		405 430 450		430	500
EPA	+		+		+	+
Acetone	—		+		+	+
Bovine serum albumin	335		410 435 455		440	~500

indole in order to detect any absorption maximum in this range. Indole did give a clear shoulder at about 360 mμ, but tryptophan did not show any such shoulder. It is probable that a low absorption maximum of tryptophan in this range may be covered by the high tail of the main absorption band around 280 mμ.

On excitation at 280 mμ in a frozen aqueous solution at 77° K the π–π u.v.

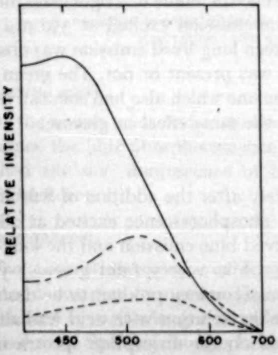

Fig. 1. (———), emission spectra of tryptophan (10^{-2} *M*) in water; (—·—), with 10^{-4} *M* dinitrophenol; (––––), with 8 *M* HCl. Excitation: 350 mμ; T = 77° K; Slit 2 mm.

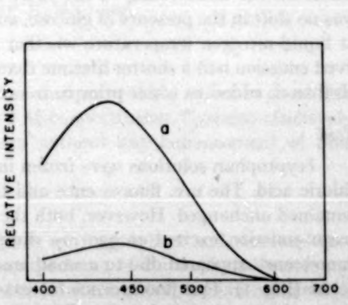

Fig. 2. Emission spectra of bovine serum albumin (10%). Excitation: 350 mμ; T = 77° K. (a) Slit 1 mm; (b) Slit 0.5 mm.

fluorescence was observed at 345 mμ with a very weak greenish long-lived emission (Fig. 3). It should be mentioned that the blue long-lived emission was not observed at this excitation[2].

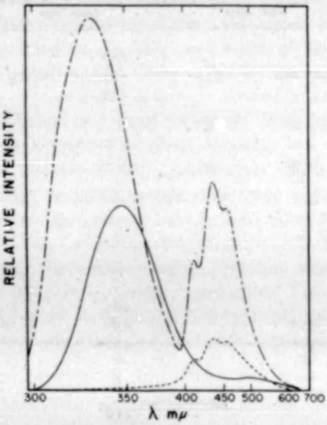

Fig. 3. (——), emission spectra of tryptophan (10^{-2} *M*) in water; (—·—), in 10% glucose; (----), in 80% acetone. Excitation: 280 mμ; T = 77° K; Slit 1 mm.

Glucose

When tryptophan was excited at 280 mμ in a frozen watery solution containing 0.5 ~ 10% glucose, not only the blue π–π phosphorescence[1] appeared at about 440 mμ, but also the u.v. fluorescence increased and shifted to 325 mμ compared with the fluorescence of a solution frozen without glucose (max. 345 mμ) (Fig. 3). The 325-mμ emission corresponded to the fluorescence of indole in a rigid glass medium EPA. On the other hand, in the short-lived blue emission excited at 350 mμ there was no shift in the presence of glucose, and the green long-lived emission was observed at liquid-nitrogen temperature whether glucose was present or not. The green long-lived emission had a shorter lifetime than the blue one which also had some structure. Methanol, added to water prior to freezing, had the same effect as glucose.

Acid

Tryptophan solutions were frozen immediately after the addition of 8 *M* hydrochloric acid. The u.v. fluorescence and the blue phosphorescence excited at 280 mμ remained unchanged. However, both the short-lived blue emission and the long-lived green emission excited at 350 mμ disappeared, while a very faint greenish-yellow fluorescence appeared due to a small amount of a secondary product to be described below (Fig. 1). This fluorescence increased when the solution with acid was allowed to stand at room temperature. The effect of acid on the absorption spectra in the near-u.v. region was also studied. On adding acid, the absorption initially decreased and then a new absorption gradually developed at 355 mμ in tryptophan (in concentrated hydrochloric acid) and at 337 mμ in indole (in 0.1 *M* HCl in methanol).

This secondary change of absorption and fluorescence was due to an irreversible reaction which occurs in an acidic medium.

Acetone

At the temperature of liquid nitrogen the short-lived blue emission excited by 350 mμ was not greatly changed by acetone. When tryptophan was excited at 280 mμ in the presence of acetone, however, the u.v. fluorescence at 345 mμ was considerably quenched without any shift of the maximum, and both blue and green long-lived emission were enhanced (Fig. 3). The same change was observed even in indole in a rigid glass medium (EPA) containing a small amount of acetone at 77° K (Fig. 4).

The decrease of the tryptophan fluorescence by acetone can be explained by *n*–*π* absorption (singlet-singlet transition) of acetone at 280 mμ, which is the wavelength used to excite tryptophan. The energy transfer between *n*–*π* triplet state of acetone formed from singlet excited state and triplet state of tryptophan may well result in the increased tryptophan phosphorescence. Such an energy transfer between triplet states was first shown by TERENIN AND ERMOLAEV[4] in a benzaldehyde (benzophenone)–naphthalene system.

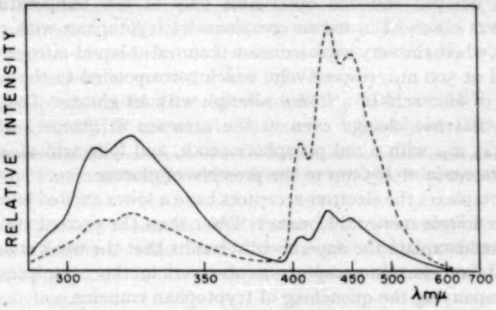

Fig. 4. (———), emission spectra of indole (10^{-2} *M*) in EPA; (– – – –), in EPA with 2% acetone. Excitation: 280 mμ; T = 77° K; Slit 1 mm.

Other amino acids

Of the amino acids so far studied, histidine was particularly active in its ability to enhance the blue phosphorescence in a 10^{-2} *M* concentration. Cysteine effectively quenched the u.v. fluorescence of tryptophan without any enhancement of blue phosphorescence.

2,4-dinitrophenol

The absorption spectra of mixed solutions of tryptophan and dinitrophenol indicated a complex formation. The absorption spectrum of dinitrophenol was found to be shifted toward a longer wave-length in the presence of tryptophan in a neutral buffer solution. Spectral changes in low concentrations of dinitrophenol in the presence of high concentrations of tryptophan indicated an association constant of about 5 l/mole. Trinitrobenzene, which is known to be a good electron-acceptor, was also found to complex with tryptophan, indole, and proteins containing tryptophan.

The change of absorption spectrum in the trinitrobenzene complex was even more marked. The measurement of difference spectra showed the absorption maximum of the trinitrobenzene-tryptophan (or indole) complex to be at about 380 mμ, while that of trinitrobenzene in itself was at 325 mμ. In recent studies[5,6], tryptophan has been found to act as an electron-donor. The formation of this type of charge-transfer complex of dinitrophenol with tryptophan in proteins may provide some insight into the mechanism of the biological action of dinitrophenol.

In the emission spectra of frozen complexes of tryptophan with nitrocompounds, all of the tryptophan emission was quenched. Instead, the complex exhibited a new weak emission at 510 mμ in the dinitrophenol-complex (Fig. 1) and at 465 mμ in the trinitrobenzene-complex at liquid-nitrogen temperatures. Dinitrophenol itself was found to emit at 557 mμ in the absence of glucose and at 510 mμ in the presence of glucose. Trinitrobenzene had the same emission at 465 mμ in a frozen solution with and without glucose. Thus, the emission of the complexes can be seen to correspond almost exactly to the luminiscence of the electron-acceptor nitro-compound. This indicates that the emission occurs from the metastable state of the electron-acceptor components. It should be made clear that the luminiscence of nitro-compounds was a delayed emission observable only at low temperature. The same phenomenon was observed in frozen complexes of tryptophan with riboflavin[5] and with folic acid[6], where the very weak emission occurred at liquid-nitrogen temperatures at 565 mμ and at 500 mμ, respectively, which corresponded to the luminiscence of riboflavin and of folic acid in a frozen solution without glucose. These emissions in the complexes did not change even in the presence of glucose, while riboflavin fluoresced at 525 mμ with a red phosphorescence, and folic acid at 425 mμ with a green phosphorescence at 485 mμ in the presence of glucose.

In these complexes the electron-acceptors have a lower excited level than tryptophan, and their lowest metastable state is lower than the excited state of the complexes[7]. This would explain the experimental results that the weak emission from the metastable state of electron-acceptors is observed in the complexes at low temperature, accompanying the quenching of tryptophan emission.

ACKNOWLEDGEMENTS

The author would like to thank Dr. A. SZENT-GYÖRGYI and Dr. I. ISENBERG for their suggestions and encouragement during the course of this work.

This research was sponsored by a grant No. H-2042R, of the National Heart Institute; a grant from the Commonwealth Fund; The National Science Foundation and the American Heart Association.

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PHOTOPHOSPHORYLATION BY SWISS-CHARD CHLOROPLASTS

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(Received August 5th, 1959)

SUMMARY

1. The preparation of chloroplasts and chloroplast fragments from swiss-chard, which were active in photophosphorylation is described. A chloroplast preparation stable for 24–48 h could be obtained.

2. A sensitive assay for the ATP formed was adopted. It allowed the measurement of very small amounts of ATP formed, and avoided several difficulties encountered in other assays.

3. The requirements in the homogenizing medium and in the reaction mixture were studied. Factors studied included: ascorbate, gas phase, pH, cofactors, magnesium, phosphate, nucleotide specificity and light intensity.

4. The chloroplasts could phosphorylate ADP, GDP, or IDP at high rates, UDP at low rates, and CDP not at all. Nucleoside diphosphokinase(s) in the preparations was demonstrated.

5. No adenyl kinase activity could be found in washed or fragmented swiss-chard chloroplasts.

6. Rates of 2000–2500 μmoles ATP formed/mg chlorophyll/h were obtained under optimal conditions, which included low chloroplast concentrations, short reaction times, appropriate ascorbate concentration, and very high light intensities (approx. 150,000 lux).

7. Confirmation was presented for the ineffectiveness of 2,4-dinitrophenol and effectiveness of ammonium ions as uncoupling agents, and the lack of exchange between inorganic phosphate and ATP in swiss-chard chloroplasts.

8. No exchange between ADP and ATP could be demonstrated.

INTRODUCTION

Photophosphorylation catalyzed by particulates isolated from higher plants was first demonstrated by ARNON *et al.*[1], and reviewed by him[2]. It is believed to be represented by the equations:

$$H_2O \xrightarrow{\text{light, chloroplasts}} (H) + (OH) \qquad (1)$$

Abbreviations to be used include: Pi, inorganic phosphate; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; GDP, guanosine diphosphate; IDP, inosine diphosphate; UDP, uridine diphosphate; CDP, cytidine diphosphate; Tris, tris-(hydroxymethyl)aminomethane; PMS, phenazine metho-sulfate; FMN, flavin mono-nucleotide; TCA, trichloroacetic acid.

$$\text{(H)} + \text{(OH)} + \text{ADP} + \text{Pi} \xrightarrow{\text{Mg, cofactor}} \text{H}_2\text{O} + \text{ATP} + \text{OH}^- \qquad (2)$$

$$\text{Total: ADP} + \text{Pi} \longrightarrow \text{ATP} + \text{OH}^-$$

Almost all the work conducted in the elucidation of the requirements, potentialities, mechanism, and function of the system utilized spinach particulates[1-10].

Several considerations prompted the present investigation with chloroplasts and chloroplast fragments from swiss-chard. It was thought desirable to make a thorough study of the photophosphorylative capacity of chloroplasts from a different plant so as to emphasize the similarities and differences which one may find in a reaction which is considered to be a basic one, common to all plants[11]. In addition, since spinach is a long-day plant[12], it will flower and terminate leaf production as soon as the critical day length is reached. This makes its growing in a greenhouse for leaf material difficult during a substantial part of the year. Swiss-chard on the other hand, is quite insensitive to day-length and can continuously supply leaves for very long periods of time. Thirdly, chloroplasts isolated from spinach possess certain disadvantages, which it was hoped, might be overcome by the use of chloroplasts isolated from a different plant. Two such disadvantages—the fast decrease of activity with storage time at 0°, and the adherence of the adenyl-kinase activity to the chloroplasts—were indeed overcome by the use of these chloroplasts.

METHODS

Preparation of chloroplasts

Chloroplasts are generally prepared by grinding the leaves by hand in a mortar and pestle, followed by differential centrifugation[1-10]. It was thought desirable to have a more standardized grinding procedure and thus several alternative methods were tried. The Virtis "45" homogenizer (Virtis Co., 160 Ashburton Ave., Yonkers, New York), under optimal conditions, was found to provide chloroplasts which were just as active as those prepared by mortar and pestle grinding, and was consequently adopted in the standard preparatory technique.

In addition, it was thought desirable to sediment the chloroplasts within the smallest range of gravity forces practicable. The usual range of 200–1000 × *g* was therefore narrowed down to 500–1000 × *g* with only a minor loss of chlorophyll containing material.

The standard procedure adopted consisted of the following stages: 5 g of a leaf, whose midrib had been removed, was cut into small pieces (approx. 1 × 1 cm in size) and put within a 100 ml capacity Virtis cup, placed in the ice water bath of the instrument. 40 ml of chilled homogenizing medium (0.4 *M* sucrose, 0.05 *M* Tris, 0.01 *M* NaCl, 0.02 *M* sodium ascorbate, pH 7.8) were added and the homogenizer run for 5 sec at 50 on its variac, followed by 25 sec with the variac set on 20. The slurry was squeezed through a double layer of cheese cloth and centrifuged at 0–2° for 90 sec at 500 × *g*. The upper, light colored, layer was sucked off and the supernatant centrifuged for 7 min at 1000 × *g*. The pellet from this centrifugation was sometimes resuspended in a small volume (2–3 ml) of homogenizing medium and used directly (unwashed chloroplasts). Usually it was washed by resuspension in 40 ml of the homogenizing medium and recentrifugation at 500 × *g* for 90 sec, followed by transfer of the supernatant and its centrifugation at 2000 × *g* for 7 min. The pellet was resuspended in 2–3 ml of homogenizing medium (once washed chloroplasts).

For the preparation of chloroplast fragments the latter pellet was resuspended in 40 ml of 10^{-2} M Tris, pH 7.8, put within the Virtis homogenizer and homogenized for 5 min with the variac set at 0, at 0–2°. The resulting suspension was centrifuged for 2 min at 2000 × g, the supernatant transferred and recentrifuged at 10,000 × g for 7 min. The pellet of the latter centrifugation was resuspended in a small volume of homogenizing medium and used (chloroplast fragments).

Reaction conditions

The standard reaction mixture contained in μmoles: Tris, pH 7.8 – 45; NaCl, 60; $MgCl_2$, 12; Na ascorbate, 30; Na, K phosphate, pH 7.8 – 12; adequate amounts of ^{32}P ($5 \cdot 10^4$–$2 \cdot 10^5$ counts/min); ADP, 12; phenazine methosulphate, 0.09, chloroplasts or chloroplast fragments containing less than 30 μg chlorophyll, and water to a total volume of 3.0 ml. The reaction was started by turning the light on, and terminated by turning the light off and adding 0.3 ml of 20 % trichloroacetic acid to the reaction mixture. The flask contents were transferred to small centrifuge tubes, and after centrifugation a sample of the supernatant was analyzed for $AT^{32}P$ as described below.

The reaction was carried out in one of two ways: (a) In an illuminated Aminco-Warburg apparatus, fitted with two rows of 150 W Mazda spot lights (4 to a row). The reaction mixture was placed in 50-ml Erlenmeyer flasks, mounted on a metal rod connected to the Warburg shaker, and shaken at 100 rev./min with an amplitude of 4 cm. For anaerobic runs, the flasks were closed with rubber stoppers fitted with glass tubing and nitrogen gas was continuously run through the flasks. The flask bottoms were approximately 17 cm from the light. Maximum light intensity at flask level in this setup was 45,000 lux. (b) For higher light intensities an aquarium was constructed (14 × 16 × 8 cm) with an inlet and an outlet for a continuous flow of water. Two of the sides were made of glass, and the aquarium was placed between two 150 W Mazda lamps, 10 cm from each other. Water flowing through it from the Warburg bath maintained the temperature throughout the experiments at 16–17°. 4 spectrophotometer cuvettes (3 ml capacity) were placed within a holder especially contructed to allow full illumination of the two optical surfaces. The holder was placed within the aquarium, and reaction started by turning the light on. Maximum light intensity in the cuvette under these conditions was 154,000 lux.

For time course studies, samples of 0.5 ml were removed at specified times and placed into test tubes containing 0.05 ml of 20 % TCA. After centrifugation, 0.3 ml of the supernatant was analysed for its $AT^{32}P$ content as described below.

Assay of ATP formation

The progress of photophosphorylation is generally followed by either measuring the change in the inorganic phosphate content of the reaction[4–7,10], or by measuring the radioactivity incorporated from inorganic ^{32}P into the organic phosphate fraction[3,8]. The first and most commonly used method possesses several disadvantages. The main ones are: (a) The initial phosphate concentration is the highest measured and thus one is starting with a high reading which decreases with time. This lowers the sensitivity and makes it very difficult to measure small amounts of Pi uptake. In addition, one cannot measure the reaction in the presence of high phosphate concentrations. (b) The addition of reducing agents, such as ascorbic acid, interfered greatly in the measurements. (c) Arsenate behaves similarly to phosphate in the color reactions involved. The method adapted overcomes all these difficulties and is still rather simple and rapid.

The assay is based on the procedure of NIELSEN AND LEHNINGER[13]: an appropriate sample of the supernatant from the TCA denatured reaction-mixture was placed within a 18 × 150 mm O.D. tube, 1.2 ml acetone added, contents mixed and allowed to stand for 10 min. The water content of the tube was made up to 2.5 ml with water saturated with a 1/1 mixture of isobutanol and benzene. 7.0 ml of a 1/1 mixture of isobutanol and benzene saturated with water was added, and the tube contents mixed. After phase separation, 0.8 ml of molybdate reagent (made by dissolving 5 g ammonium molybdate in 40 ml of 10 N H_2SO_4 and making it up to 100 ml with water) were added to the side of the tube, the water layer gently mixed, and allowed to stand for 5 min. Each tube was now vigorously mixed for 30 sec. After phase separation the upper layer was sucked off into a trap. 0.02 ml of 0.02 M KH_2PO_4 were added to the aqueous layer, followed by 7.0 ml of the isobutanol–benzene and the tube contents vigorously mixed for 30 sec. After phase separation the upper layer was again sucked off and 1.0 ml of the water phase placed in a stainless-steel planchet and counted directly.

All mixings throughout the procedure were made with a glass rod thickened at one end to form a small ball whose diameter was slightly smaller than the diameter of the glass tube. Moving this rod up and down the tube provided very good mixing of phases and avoided the necessity of using separatory funnels.

For each reaction two extra samples were counted. One was a zero-time control for the reaction mixture. This value was substracted from all counts/min to give the corrected counts/min. The second was a sample of the supernatant from the TCA treated reaction mixture, diluted to 1.0 ml with water saturated with isobutanol–benzene, and counted directly to give the total counts/min introduced. The μmoles of ATP formed can be calculated from the following equations:

$$\mu\text{moles ATP formed} = (\text{corr. counts/min in planchet}) \times 3.3 \times \frac{(\text{volume in flask after TCA addition})}{(\text{volume used for assay})} \times \frac{(\mu\text{moles Pi in flask})}{(\text{total counts/min in flask})}$$

It was found initially that significant amounts of counts could be recovered in the zero-time sample (0.1–0.2 % of the total introduced). This could be completely overcome by first treating the ^{32}Pi as it was received in 1N HCl at 100° for 1 h, followed by the addition of acid washed charcoal and filtration. After such treatment zero-time values decreased to 0.01–0.02 % which was completely negligible in the standard assay.

Chlorophyll was determined by the modification of ARNON[16].

Materials

Phenazine methosulfate was synthesized by the method of DICKENS AND McILLWAIN[14] according to directions kindly supplied by Dr. T. P. SINGER. All other chemicals were commercial preparations.

Swiss-chard (*Beta vulgaris*) was grown in pots in a greenhouse. 10–20 plants supplied sufficient leaves for several months of work.

RESULTS

Preparatory requirements

It was noticed early in the preparation of chloroplasts from swiss-chard that when the leaves were homogenized in the sucrose–Tris–NaCl mixture used for spinach[10] the homogenate would rapidly darken. In addition the swiss-chard chloroplasts lost their activity very fast upon standing at 0°. Both of these effects could be overcome by the inclusion of ascorbate in the homogenizing medium. Table I illustrates the stability with time, shown by chloroplasts prepared in this manner. Although not all preparations maintained activity for such long periods, they will usually maintain it for 24–48 h. 0.02 *M* ascorbate seemed optimal and was thus adopted in the standard preparative technique.

Since spinach chloroplasts never showed such stability, a comparative experiment was run using spinach and swiss-chard chloroplasts prepared in the identical manner and tested together for their activity. Table II shows that the effect was indeed, much more marked in swiss-chard chloroplasts. In the absence of ascorbate spinach chloroplasts lost activity slowly, as shown earlier[10], while chloroplasts prepared from swiss-chard lost it rapidly. The addition of ascorbate to the homogenizing medium had a marked effect in the swiss-chard chloroplasts but small to no effect in spinach.

TABLE I

EFFECT OF ASCORBATE IN HOMOGENIZING MEDIUM

Reaction conditions as described under METHODS. Reaction time, 15 min. Light intensity approximately, 40,000 lux. Chloroplasts once washed. Chlorophyll 12–18 μg/ml. Temperature, 25° gas phase–air. Chloroplasts kept at 0° in the dark between runs. All reaction mixtures contained a total of 10 μmoles ascorbate/ml.

Ascorbate in medium	*Time after homogenization at which activity was tested*					
	Hours					
M	*1*	*23*	*46*	*70*	*94*	*214*
	μmoles ATP formed/mg chlorophyll/h					
0	230	64	0	20	0	0
0.01	368	362	336	356	274	128
0.02	496	508	468	464	386	164
0.04	432	396	370	386	302	178

TABLE II

EFFECT OF ASCORBATE IN HOMOGENIZING MEDIUM-SPINACH *vs.* SWISS-CHARD

Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity approximately, 40,000 lux. Chloroplasts once washed. Chlorophyll: Spinach, 14–15 μg/ml, swiss-chard-6–7 μg/ml. Temperature, 25°. Gas phase-air. Chloroplasts kept at 0° in the dark between runs. All reaction mixtures contained a total of 10 μmoles ascorbate/ml.

Source of chloroplasts	*Ascorbate in medium*	*Hours after homogenization at which activity was tested*		
		0.5	*23.5*	*56*
	M	*μmoles ATP formed/mg chlorophyll/h*		
Swiss-chard	0	272	14	2
Swiss-chard	0.02	356	342	165
Spinach	0	297	165	6
Spinach	0.02	218	117	35

Occasionally spinach chloroplasts showed some response to ascorbate in the homogenizing and storage medium, but the effect was always small and the activity continuously decreased in all cases.

The stability of swiss-chard chloroplasts prepared in the sucrose–Tris–NaCl–ascorbate medium was also emphasized by the maintainance of activity during washing and subfragmentation. Table III shows that 3 washes or subfragmentation did not change the activity per unit chlorophyll of the preparation.

TABLE III

EFFECT OF WASHING AND SUBFRAGMENTATION

Reaction conditions as described under METHODS. Reaction time: Expt. 1, 30 min, Expt. 2, 15 min. Light intensity approximately 40,000 lux. Chlorophyll: Expt. 1, 7–11 μg/ml; Expt. 2, 10–12 μg/ml. Temperature, 25°. Gas phase–air.

Experiment	*Treatment*	*μmoles ATP formed/mg chlorophyll/h*
1	No wash	392
1	One wash	380
1	Three washes	372
2	One wash	456
2	Subfragmented	460

It could be mentioned at this point that microscopically the chloroplast preparation can be seen to be composed mainly of whole chloroplasts. The fragmented chloroplasts have the appearance of small oval shaped dark particles of a rather definite size. The latter preparation contains, however, a few whole chloroplasts. Both preparations contain a small amount of cell fragments of indefinite shape, other than chloroplasts or their fragments.

Requirements in medium

Ascorbate: In addition to the requirement for ascorbate in the homogenizing medium, it soon became apparent that its addition to the reaction mixture markedly increased the rates of phosphorylation. Closer examination of the latter effect revealed that it was strongly time-dependent. Fig. 1 illustrates a time course study in the presence of increasing concentrations of ascorbate. It can be seen that at very short time intervals the addition of ascorbate had no significant effect. However, if the reaction was allowed to proceed for longer time periods the effect became very marked. Ascorbate seemed to act in maintaining the activity of the chloroplasts for longer time periods. It should be noted that the activity of ascorbate is rather specific. It is not at all replaced by other reducing agents such as glutathione or cysteine. In addition, the requirement for ascorbate is just as pronounced with fragmented chloroplasts as it is with whole ones (see Fig. 3), indicating that whatever it acts on is very closely associated with the enzymic complex responsible for photophosphorylation.

The time-dependent requirement for ascorbic acid in the medium is just as apparent, in our experience, with spinach or swiss-chard chloroplasts.

Gas phase: Fig. 2 illustrates a time-course study of the reaction under nitrogen or air, in the presence or absence of ascorbate. It is clear that nitrogen and ascorbate act in a similar manner, prolonging the maintainance of activity in the chloroplasts. Under nitrogen ascorbate has little to no effect.

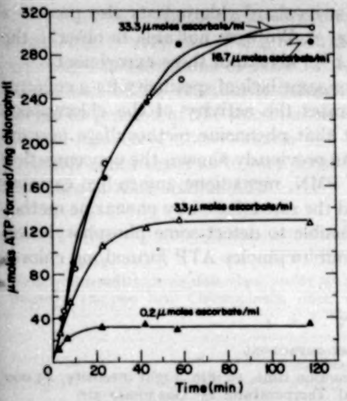

Fig. 1. Effect of ascorbate in the reaction mixture. Reaction conditions as described under METHODS. Light intensity approximately 40,000 lux. Chloroplasts once washed. Chlorophyll, 11 μg/ml. Temperature 25°. Gas phase–air.

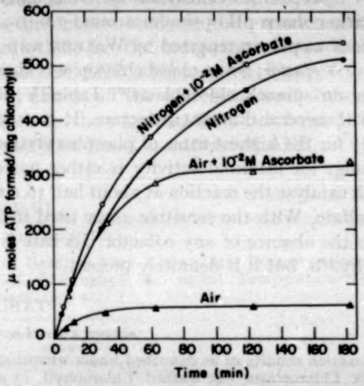

Fig. 2. Effect of ascorbate in the reaction mixture; Nitrogen *vs.* Air. Reaction conditions as described under METHODS. Light intensity, 45,000 lux. Chloroplast fragments. Chlorophyll, 2.9 μg/ml. Temperature, 15°.

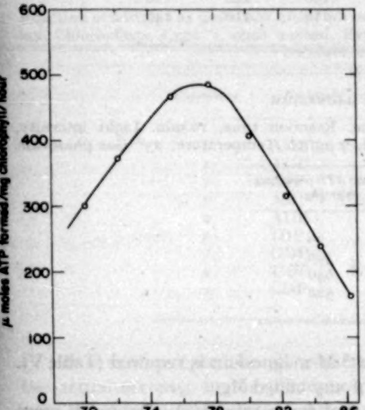

Fig. 3. pH curve. Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity, 40,000 lux. Chloroplasts, once washed. Chlorophyll, 11 μg/ml. Temperature, 25°. Gas phase–air. pH changed by varying the pH of the Tris added. The pH of the reaction mixture was measured at the end of the reaction.

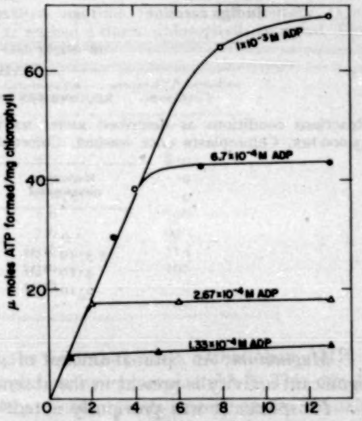

Fig. 4. Requirement for ADP. Reaction conditions as described under METHODS. Light intensity, approximately 40,000 lux. Chloroplasts, once washed. Chlorophyll, 11 μg/ml. Temperature, 25°. Gas phase–air.

pH: As previously shown for spinach[15], swiss-chard chloroplasts also possess a rather sharp pH optimum around 7.6–7.9 (Fig. 3). We were not able to observe the peak at pH 7.0 reported by WALKER AND HILL[9] in several of these experiments.

Cofactor: Swiss-chard chloroplasts show the same lack of specificity for a cofactor as do spinach chloroplasts[10]. Table IV illustrates the activity of the chloroplasts with several different cofactors. It is evident that phenazine methosulfate permits by far the highest rates of phosphorylation. As previously shown, the concentration range for maximal activity is rather narrow. FMN, menadione and indigo carmine all catalyse the reaction at about half to a third the rate allowed by phenazine methosulfate. With the sensitive assay used it is possible to detect some phosphorylation in the absence of any cofactor. Its rate is about 10 μmoles ATP formed/mg chlorophyll/h, but it is definitely present.

TABLE IV

ACTIVITY WITH SEVERAL COFACTORS

Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity, 45,000 lux. Chloroplasts, one washed. Chlorophyll, 15 μg/ml. Temperature, 25° Gas phase–air.

Cofactor	*Concentration (M)*				
	$1 \cdot 10^{-5}$	$3.3 \cdot 10^{-5}$	$1 \cdot 10^{-4}$	$3.3 \cdot 10^{-4}$	$1 \cdot 10^{-3}$
	μmoles ATP formed/mg chlorophyll/h				
PMS	428	712	366	340	34
FMN	194	272	358	346	310
Menadione	186	304	290	246	120
Indigo carmine	194	226	216	210	140

TABLE V

REQUIREMENT FOR MAGNESIUM

Reactions conditions as described under METHODS. Reaction time, 10 min. Light intensity, 45,000 lux. Chloroplasts once washed. Chlorophyll, 7 μg/ml. Temperature, 25° Gas phase–air.

Magnesium concentration	*μmoles ATP formed/mg chlorophyll/h*
0	70
$5.3 \cdot 10^{-4}$ *M*	520
$4 \cdot 10^{-3}$ *M*	640
$2 \cdot 10^{-2}$ *M*	520

Magnesium: An optimal amount of $4 \cdot 10^{-3}$ *M* magnesium is required (Table V). significant activity is present in the absence of any added Mg.

Phosphate: It was previously noted[15,10] that for maximal activity much more phosphate was required than the amount necessary for ATP production. With the assay employed this could be retested with greater ease. Table VI illustrates the continuously increasing rates obtained with increasing phosphate concentrations. Higher phosphate concentrations could not be conveniently used at present because of assay difficulties. In all cases the uptake of phosphate was not greater than 10 % of the total phosphate present in the reaction mixture. The high rates shown were due to the high light intensity and the short reaction time (see below).

Nucleotide: In contrast with phosphate, the ADP concentration required for saturation is very low. Fig 4 illustrates a time course study in the presence of increasing concentrations of ADP. It is clear that, for the concentrations used, the initial rate of the reaction is independent of the ADP concentration. The reaction seems to proceed at the maximal rate until almost all the ADP is utilized. In view of the absence of adenyl-kinase and ATPase in these preparations (see below) the reaction could serve as a very sensitive assay for small quantities of ADP, in the presence of AMP and/or ATP.

TABLE VI

REQUIREMENT FOR PHOSPHATE

Reaction conditions as described under METHODS. Reaction time, 2 min. Light intensity approximately 100,000 lux. Chloroplasts, once washed. Chlorophyll, 8.6 μg/ml. Temperature, 16°. Gas phase–air.

Phospahte concentration	*μmoles ATP formed/mg chlorophyll/h*
$1.4 \cdot 10^{-3}\ M$	900
$4.2 \cdot 10^{-3}\ M$	1004
$8.4 \cdot 10^{-3}\ M$	1210
$1.7 \cdot 10^{-2}\ M$	1290

TABLE VII

NUCLEOTIDE SPECIFICITY

Reaction conditions as described under METHODS. Reaction time, 10 min. Light intensity, 45,000 lux. Chloroplasts, Expt. 1, once washed, Expt. 2, washed 3 times. Chlorophyll: 19 μg/ml. Temperature, 25°. Gas phase–air.

Experiment	*Nucleotide*	*Concentration μmoles/ml*	*μmoles ATP formed/mg chlorophyll/h*
1	AMP	6	0
1	ADP	6	420
1	ATP	0.7	0
1	AMP + ATP	6 + 0.7	0
2	ADP	1.6	796
2	IDP	1.6	640
2	GDP	1.6	514
2	UDP	1.6	196
2	CDP	1.6	2

It is now accepted by all workers in the field that ADP, rather than AMP, is the actual acceptor in the photophosphorylation by chloroplasts[3,6,10]. This can be very beautifully demonstrated with swiss-chard chloroplasts since they are virtually free of adenyl kinase activity. Table VII, experiment 1 shows that even with once washed whole chloroplasts, activity is seen only in the presence of ADP. This is in striking contrast with spinach chloroplasts, where only after exhaustive washings can one achieve a relatively adenyl-kinase free preparation[3,10,15].

The specifity of the reaction for nucleosides diphosphate other than ADP, has not been thoroughly tested. KRALL *et al.*[6] reported that UDP, GDP, IDP and CDP can be phosphorylated by spinach chloroplasts, but at lower rates than ADP.

Table VII, experiment 2, illustrates the activity of swiss-chard chloroplasts with these phosphate acceptors. It is evident that thrice washed chloroplasts were capable of phosphorylating IDP and GDP at almost as fast a rate as ADP. UDP was utilized at a lower rate, while CDP was inactive. The same results were obtained with fragmented chloroplasts.

The question arose whether the results were a reflection of the specificity of the enzyme combining with ADP. It could also be consequence of the esterification of small amounts of contaminating ADP in the preparations of the other nucleosides diphosphate, followed by nucleoside diphospho–kinase activity. The latter possibility was tested in the following manner: chloroplasts were incubated in the usual reaction mixture and allowed to synthesize $AT^{32}P$ in the light. After practically all of the ADP was phosphorylated, the light was turned off and another nucleoside diphosphate added to the reaction mixture. The reaction was allowed to proceed for another 30 min in the dark, after which denaturation and paper chromatography was carried out. The radioactivity distribution between the adenine nucleotide spot and the other nucleotide in question at the end of the reaction is recorded in Table VIII. If no nucleoside diphosphokinase activity was present all the radioactivity should have been recovered in the $AT^{32}P$ spot. However, it is seen that in the case of IDP, GDP, and UDP a substantial part of the radioactivity has been transferred to their triphosphates. In the case of CDP, however, only a small percentage has been transferred. This percentage might even be accounted for by other contaminating nucleotides in the CDP preparation. The relative inactivity of the nucleoside diphosphokinase for CDP is in agreement with the inability of the chloroplasts to phosphorylate CDP.

TABLE VIII

NUCLEOSIDE DIPHOSPHOKINASES IN CHLOROPLASTS

Reaction conditions as described under METHODS. Reaction time: light, 10 min. Dark, 30 min. Light intensity, 40,000 lux. Chloroplasts, once washed. Chlorophyll, 25 μg/ml. Temperature, 25°. Gas phase–air. See text for details. One dimensional paper chromatography according to the method of BERGKVIST AND DEUTSCH[20], with saturated ammonium sulfate, water, isopropanol (79 : 19 : 2; v/v) as the developing agent.

Nucleotides in dark reaction	*Radioactivity distribution % of total in*	
	ATP	*Other nucleotide*
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$	100	0
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* IDP	63	37
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* GDP	61	39
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* UDP	59	41
$1.3 \cdot 10^{-3}$ *M* $AT^{32}P$ + $1.3 \cdot 10^{-3}$ *M* CDP	88	12

These data do not allow a definite conclusion as to the specificity of the nucleotide combining enzyme of photophosphorylation. They seem to indicate, however, that the activity observed with nucleotides other than ADP may be accounted for by the nucleoside diphosphokinase(s) present in the chloroplasts. The preparation of chloroplast fragments, free of the latter enzyme, is being attempted. Such a preparation would provide means of testing the specificity of the nucleotide combining enzyme of photophosphorylation.

Light: As soon as light intensity studies were begun it became apparent that the intensity of 45,000 lux used, on the basis of the requirements previously determined for spinach chloroplasts[10], was not sufficient. Fig. 5 illustrates the light intensity requirements as measured with the set up described under methods (b). It is evident that the reaction was not approaching saturation until at least 100,000 lux were supplied. Complete saturation does not seem to have been attained even at 154,000 lux, the limiting intensity with that apparatus. The obvious result of this finding was the extremely high rates of photophosphorylation recorded. Under 154,000 lux of light we have continuously observed rates of 1300–2000 μmoles phosphate esterified /mg chlorophyll/h.

Several other factors emerge as important in the attainment of these high rates. As can be seen in all time-course curves presented, the activity in the preparations is not linear with time. Even under the best conditions attained to date, the rate continuously decreased as time proceeds. It is thus important to measure the reaction at short intervals for maximal rates. A second factor can be seen in Fig. 5. Although it was previously found that the effect of ascorbate in the reaction mixture is apparent only at longer time intervals, it is clear that it is also dependent on the intensity of the light supplied. Thus in a 5-min reaction (Fig. 5), ascorbate has little to no effect up to about 40,000 lux, but the effect is increasingly marked thereafter. A third factor can be seen in Fig. 6. It illustrates chloroplast concentration curves measured at several light intensities. The statement is often made on the basis of one such curve that the concentration of chloroplasts used was below the limit of light saturation (see, for example, ref.[3]), meaning in the lowest portion of the curve illustrated. Although this is reasonably sound, Fig. 7 illustrates that even at the lowest portion of the curves, at chlorophyll concentration of 3 μg/ml, light saturation was certainly not reached until 154,000 lux were supplied. It is therefore important to run a light concentration curve of the type shown in Fig. 5 to determine the level of light saturation under the particular conditions in question.

At the lowest chlorophyll concentrations, highest light intensity, in a 2-minute reaction time, and with optimal ascorbate concentration the data of Fig. 6 represents a specific activity of 2500 μmoles ATP formed/mg chlorophyll/h.

Inhibitors

Arsenate: It has previously been shown that arsenate was a competitive inhibitor for phosphate in photophosphorylation by spinach chloroplasts[17]. Since the strictly competitive nature of the effect was valuable as a tool in several investigations concerning the mechanism of ATP formation[17, 18], it was thought important to confirm it with chloroplasts isolated from swiss-chard. Fig. 7 represents a LINEWEAVER-BURK plot of the effect of arsenate on the reaction. It is evident that here, too, arsenate inhibits ATP formation only by virtue of its competition with phosphate.

Uncouplers: The classical uncoupler of phosphorylation from electron transport, 2,4-dinitrophenol, was shown to be ineffective in uncoupling the non-cyclic photophosphorylation of spinach chloroplasts[18], and effective in inhibiting its cyclic phosphorylation only at high concentrations[4, 19]. As indicated in Table IX, swiss-chard chloroplasts were even less sensitive than spinach to dinitrophenol. Whereas spinach chloroplasts were inhibited 60–80 % by 10^{-3} M dinitrophenol[4, 19], only 20 % inhibition is seen with the swiss-chard preparation.

By contrast, ammonium ions, which were previously shown to uncouple noncyclic

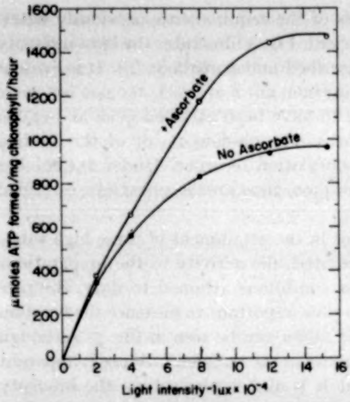

Fig. 5.

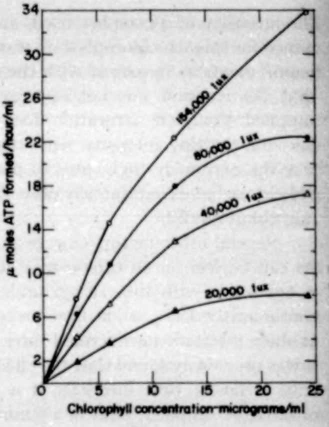

Fig. 6.

Fig. 5. Requirement for light intensity. Reaction conditions as described under METHODS. Reaction time, 5 min. Light intensity, as marked. Chloroplasts, once washed. Chlorophyll, 7 µg/ml. Temperature 16°. Gas phase–air.

Fig. 6. Effect of chloroplast concentration at several light intensities. Reaction conditions as described under METHODS. Reaction time, 2 min. Light intensity, as marked. Chloroplasts, once washed. Chlorophyll, as marked. Temperature 16°. Gas phase-air.

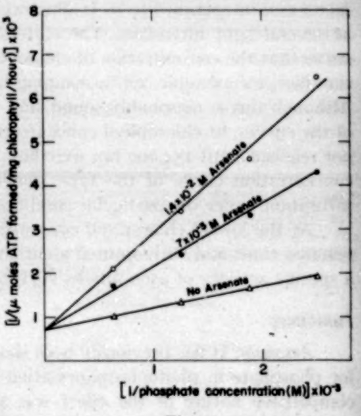

Fig. 7.

Fig. 7. Arsenate as a competitive inhibitor. Reaction conditions as described under METHODS. Reaction time, 5 min. Light intensity 40,000 lux. Chloroplasts, once washed. Chlorophyll, 6.6. µg/ml Temperature, 16°. Gas phase–air.

photophosphorylation[1],[8] and inhibit cyclic phosphorylation[4] of spinach preparations, behave in the identical manner with swiss-chard chloroplasts. Table IX ilustrates the strong inhibition produced by ammonium chloride. The concentration required for 50 % inhibition was about $6 \cdot 10^{-4}$ *M*, and the inhibition seemed to be directly proportional to the logarithm of the ammonium ion concentration.

Mechanism

Pi-ATP exchange and ATPase: The surprising lack of ATPase or Pi-ATP-exchange in spinach chloroplasts was previously shown[17]. It is here strongly confirmed with chloroplasts from swiss-chard. Table X illustrates the complete lack of any reaction

TABLE IX

EFFECT OF 2,4-DINITROPHENOL AND AMMONIUM CHLORIDE

Reaction conditions as described under METHODS. Reaction time, 5 min. Light intensity, 45,000 lux. Chloroplasts, once washed. Chlorophyll: dinitrophenol experiment, 4.8 μg/ml; NH_4Cl experiment, 6.6 μg/ml. Temperature, 25°. Gas phase–air.

Inhibitor	*Concentration M*	*μmoles ATP formed/mg chlorophyll/h*	*% of control*
2,4-dinitrophenol	0	625	(100)
2,4-dinitrophenol	$1.0 \cdot 10^{-5}$	630	101
2,4-dinitrophenol	$1.0 \cdot 10^{-4}$	700	112
2,4-dinitrophenol	$1.0 \cdot 10^{-3}$	500	80
NH_4Cl	—	630	(100)
NH_4Cl	$5.0 \cdot 10^{-4}$	375	60
NH_4Cl	$1.3 \cdot 10^{-3}$	186	30
NH_4Cl	$5.0 \cdot 10^{-3}$	15	2

TABLE X

LACK OF Pi-ATP EXCHANGE AND ATPASE

Reaction mixture contained the usual components (see METHODS), except for ADP and inorganic phosphate being replaced by the materials indicated under vessel contents. Reaction performed in test tubes in the dark, with once washed chloroplasts. Total volume, 1.0 ml. Chlorophyll, 25 μg/ml. Temperature, 25°. Gas phase–air. In the first and second experiments samples of 0.2 ml were taken out at the indicated times, and transferred into tubes containing 0.02 ml of 20% trichloroacetic acid. The samples were applied to Whatman No. 1 paper and chromatographed by the method of BURROWS *et al.*[21], using acetone, 15% trichloroacetic acid (65:35, v/v). Samples of the third experiment were analyzed as described under METHODS. The $AT^{32}P$ used was synthesized with swiss-chard chloroplasts, adsorbed on charcoal, and extracted from it with 50% ethanol, 0.2% NH_4OH. The extract was applied to Whatman No. 1 paper and developed by the method of KREBS AND HEMS[22], using isobutyric acid–concentrated ammonium hydroxide–water (66:1:33, v/v). After drying, the $AT^{32}P$ strip was cut out and eluted with water.

Vessel contents	*Reaction time (min)*	*% of total radioactivity in*	
		ATP	*Pi*
1 μmole $AT^{32}P$	0	81	19
	60	80	20
1 μmole $AT^{32}P$ + 2 μmoles Pi	0	82	18
	60	82	18
1 μmole ATP + 4 μmoles ^{32}Pi	0	0	100
	60	0	100

in 60 min, which was sufficient to phosphorylate, under the same conditions, many times the amount of ATP supplied.

The addition of ADP and changes in the reaction components did not alter the results.

ADP–ATP exchange: Since these chloroplasts are free of adenyl kinase, they provide excellent material for the study of the exchange between ADP and ATP. This exchange was found in mitochondria[23] and was related to the last step in oxidative phosphorylation. Its rate was several fold faster than that of the Pi–ATP exchange, which in itself is many fold faster than the phosphorylating reaction.

The data of Table XI illustrate the complete lack of such an exchange in photophosphorylation by swiss-chard chloroplasts. The absence of adenyl kinase is indicated by the lack of any simultaneous increase in the AMP and ATP in any of the experiments. This was true even when the reaction was run with washed chloroplasts. The third experiment indicates that, in the light, the reaction conditions used were sufficient to completely esterify the ADP supplied in 10 min. However, in the dark, even 60 min of reaction did not show any measurable exchange between ADP and ATP under the identical conditions.

TABLE XI

LACK OF ADP–ATP EXCHANGE

Reaction mixture contained the usual components (see METHODS) except for ADP and inorganic phosphate being replaced by the materials indicated under vessel contents. In addition, all vessels contained $2 \cdot 10^{-3}$ *M* ATP. The reaction was carried out in small test tubes, with chloroplast fragments. Total volume, 0.3 ml. Chlorophyll, 29 μg/ml. Temperature, 25°. Gas phase–air. Samples of 0.05 ml were taken at the indicated times and transferred into tubes containing 0.005 ml of 20% trichloroacetic acid. The samples were applied to Whatman No. 1 paper, together with a marker for AMP, and chromatographed by the method of KREBS AND HEMS[22], using isobutyric acid–concentrated ammonium hydroxide–water (66 : 1 : 33, v/v). After thorough drying, the spots were marked under an u.v. lamp, cut out and their radioactivity determined in a gas flow counter.

Vessel contents	*Illumination (lux)*	*Reaction time (min)*	*% of total counts in*		
			AMP	*ADP*	*ATP*
$2 \cdot 10^{-3}$ *M* [^{14}C]ADP	None	0	9	82	9
		60	11	79	10
$2 \cdot 10^{-3}$ *M* [^{14}C]ADP + $5 \cdot 10^{-3}$ *M* Pi	None	0	9	82	9
		60	8	83	9
$2 \cdot 10^{-3}$ *M* [^{14}C]ADP + $5 \cdot 10^{-3}$ *M* Pi	45,000	0	10	81	9
		10	8	6	86

DISCUSSION

The effects of ascorbate in the photophosphorylation system have been a source of continuous and contradicting reports. It was first reported by ARNON and his group[1] that it was essential for maximal rates of photophosphorylation. AVRON, JAGENDORF AND EVANS[15] confirmed the requirement, but observed no such need when phenazine methosulfate was used as a cofactor[10]. ARNON *et al.*[24] reported that the inclusion of 0.01 *M* ascorbate in the preparatory medium increased the stability of spinach chloroplasts. OHMURA[4] confirmed both effects of ascorbate, stabilizing and increasing the rates of photophosphorylation. He further showed that the increase in rate was apparent only when the reaction was carried out for longer periods of time. WESSELS[5] also observed the ascorbate and time dependent increase of rate. In addition he reported that the effect was absent when the reaction was run under anaerobic conditions.

All of these observations are much more clearly seen with the swiss-chard chloroplasts as demonstrated under results. It would seem that where the effect of ascorbate was not observed, short time periods were used[10]. The increase in rate due to ascorbate was not, therefore, cofactor dependent, as it seemed at first[10]. It is, however, time dependent (Fig. 1), and therefore when a cofactor permitting higher rates was used, shorter time intervals were required and the response could not be seen. However, under very high light intensities, and consequently very high rates, a requirement for ascorbate was apparent even at short time intervals.

It is difficult to define the mode of action of ascorbic acid. Attempts to relate its effect to an inhibitory action of polyphenol oxidase, which can be demonstrated in these preparations, have not succeeded. Although sodium diethyl dithio carbamate, a potent inhibitor of the latter enzyme, causes an increase in the rate of photophosphorylation, it does so independent of the presence of ascorbate. One can only say that both actions of ascorbate can be attributed to a stabilizer, rather than a cofactor function. It is also clear that this stabilizing action is related to its ability to prevent or correct some deleterious oxidative effect, since no increase of rate was observed under anaerobic conditions (Fig. 2).

The requirements in the medium for swiss-chard chloroplasts were, in general, quite similar to the ones previously described for chloroplasts from spinach. The high concentration of phosphate needed for saturation was strongly confirmed with these chloroplasts. Although no explanation can, as yet, be given to this phenomenon, it is especially striking when compared to the very high affinity of the same chloroplasts for ADP.

The presence of nucleoside diphosphokinase(s) in the chloroplasts, makes it difficult to reach a conclusion regarding the final identification of the primary phosphate acceptor. The data presented, however, would place ADP or GDP as the only likely candidates.

Most workers in the field seem to employ relatively low intensity light for the reaction[3–5,8]. Although light is recognized by all to be an essential component, it was rarely given a thorough study, and its intensity is often expressed in non-comparable terms. It is clear, however, from the results presented herewith, and earlier[10], that under optimal conditions it often was the limiting factor in the reaction. The rates reported here of 2000 μmoles ATP formed per mg chlorophyll per h, could be obtained only at very high intensities around 150,000 lux. It might be worthwhile to call attention again to the inconclusiveness of chloroplast concentration curves, when interpreted as indicating saturation by light (see Fig. 7 and text under RESULTS).

The continuous increase in maximal rates of photophosphorylation as obtained by different workers in the field was quite remarkable. In units of μmoles ATP formed/mg chlorophyll/h, the initial observations[1] reported rates of 3, which were improved by the addition of FMN or menadione to 34. Close study of the requirements of the system resulted in another increase up to a rate of 200[15]. ALLEN *et al.*[3] upon further study, obtained rates of 360–510. Rates in the same order of magnitude were also reported by OHMURA[4]. The use of phenazine methosulfate as a cofactor[10], resulted in rates of 700–900, and in this report the rate has been boosted another 2–3 fold to 2000–2500. It was obtained as a result of a higher light intensity, higher phosphate concentration, lower chloroplast concentration, a very short reaction time, and a sensitive assay permitting the accurate measurement of the small amounts of ATP formed.

The demonstration of no exchange between ADP and ATP emphasizes again the difference between the mechanisms of oxidative and photophosphorylations[17]. It is in agreement with the mechanism previously proposed[17,25] and a consequence of the irreversibility of such a step as has been predicted from earlier, unrelated data[17].

ACKNOWLEDGEMENT

The able technical assistance of Mrs. F. ITZHAK is gratefully acknowledged.

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THE ROLE OF THE SULFHYDRYL GROUPS IN THE STABILISATION OF THE STRUCTURE OF THE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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(Received July 20th, 1959)

SUMMARY

The effect of the blocking of the SH groups on the spatial structure of the swine muscle D-glyceraldehyde-3-phosphate dehydrogenase was studied. The treatment with *p*-chloromercuribenzoate resulted in significant changes in the optical rotation and the intrinsic viscosity of the protein, the D-glyceraldehyde-3-phosphate dehydrogenase molecule being altered in the direction of denaturation. From these data it is assumed that the SH groups may play an important role in the stability of the secondary structure of the enzyme.

Abbreviations: PGAD = D-glyceraldehyde-3-phosphate dehydrogenase; DPN = diphosphopyridine nucleotide; PCMB = *p*-chlormercuribenzoate.

INTRODUCTION

In an earlier paper it was described[1] that the diphosphopyridine nucleotide coenzyme plays a significant role in the stabilisation of the secondary structure of the PGAD molecule. Szabolcsi *et al.*[2] have shown that in the case of PGADs isolated from various mammals also, the blocking with PCMB abolishes the steric hindrance which the native protein structure exerts against proteolytic breakdown. On the other hand it was reported that the digestion of PGADs by trypsin is inhibited by the presence of excess DPN[3,4].

Velick[5] has shown that the rabbit muscle PGAD contains no disulfide bonds and he assumes, that various ions and salts play an important role in the stabilisation of the PGAD molecule[6].

METHODS AND MATERIALS

Enzyme preparation

The enzyme used in the experiments was isolated from swine muscle, according to the method described earlier[7]. Three different preparations of four times recrystallized PGADs were examined. Before testing the proteins were dialysed at 2 to 4° against several changes of distilled water and against a 0.1 *M* phosphate buffer of pH 8.4. The protein content of the dialysed solutions was determined spectrophotometrically.

Treatment with PCMB

The samples containing 0.5 to 1.0 % protein were treated with 1 to 20 equiv. (µmole PCMB per µmole protein) of PCMB and the optical rotation and viscosity were measured.

Measurement of the optical rotation

A Schmidt & Haensch type precision polarimeter (accuracy: ± 0.01 degrees) with a Zeiss Na lamp as a light source was used. The values given are an average of 9 to 15 readings at 20.0°. The results for the different samples agreed within 2 %.

Viscosity measurements

Capillary viscosimeter of the Ostwald type (outflow with distilled water: 65 and 73 sec respectively) was used.

Intrinsic viscosity $[\eta]$ was determined on the basis of the correlation

$$[\eta]_{c \to 0} = \frac{(\eta_{\mathrm{rel}} - 1)}{c} \times 10^2,$$

where c is the protein concentration in g/100 ml, plotting the $(\eta_{\mathrm{rel}} - 1)/c$ values against the protein concentration. An ultrathermostat of the Höppler type served to ensure constancy of temperature. In every test the temperature was 20.0°, varying within ± 0.05°.

RESULTS

Optical rotatory studies

After addition of PCMB, the rotatory power of the protein solution increases in the negative direction for an interval of 5 to 15 min, in proportion to the quantity of PCMB present, then becomes constant (Fig. 1). After 20 to 25 min an opalescence is noticeable, and a few minutes later a precipitate is formed, depending in intensity on the concentration of PCMB. The values measured after 15 min incubation, are plotted against PCMB concentration (Fig. 2). It can be seen, that the changes in the rotatory power are linear with the quantity of PCMB present, within the range of 1 to 15 equiv. Further amounts of PCMB added do not increase any more the laevorotation of the protein solution.

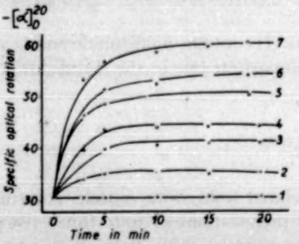

Fig. 1. The effect of various amounts of PCMB on the specific rotation of the swine muscle PGAD. Curve 1 is the control, curves 2 to 7 represent the effect of 2, 5, 7, 10, 12 and 15 equiv. of PCMB respectively.

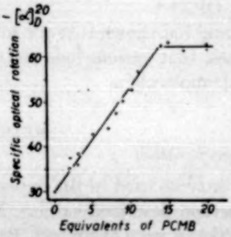

Fig. 2. Specific optical rotation of the swine muscle PGAD as a function of the PCMB concentration.

According to PAULING AND COREY[8] the increased rotation in the negative direction results from a disintegration of the right handed helix structure. The extent of this disintegration or transition (f_h) may be characterized by the following correlation, as suggested by HARRINGTON AND SCHELLMAN[9].

$$f_h = \frac{[\alpha]_{unf} - [\alpha]_{measured}}{[\alpha]_{unf} - [\alpha]_{fold}}$$

where $[\alpha]_{fold}$ is the specific rotation of the folded protein, in the present case — 30.6°. The value $[\alpha]_{unf}$ is the specific rotation characteristic for the completely unfolded protein, which contains no helix structure, in the case of PGAD denatured with 8 *M* urea being —99.7°. The f_h values are plotted against PCMB concentration (Fig. 3).

The data indicate that after all the sulfhydryl groups of the swine muscle PGAD

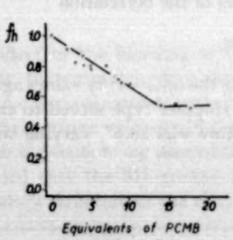

Fig. 3. PCMB induced transition (f_h) of the swine muscle PGAD.

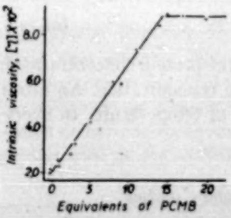

Fig. 4. Variation of intrinsic viscosity of the swine muscle PGAD with the PCMB concentration.

(14-SH/mole) has been blocked[3], a considerable part of the helix structure was disintegrated as calculated from the changes in the optical rotation.

Viscosity studies

When measuring viscosity changes in time had not been followed, the testing was usually made 1 to 2 h after the addition of PCMB. When necessary, the precipitate formed had previously been removed and the protein contents were corrected accordingly. The curve in Fig. 4 shows the changes in the intrinsic viscosity as the function of the PCMB concentration. The intrinsic viscosity of the protein solution increases in proportion to the quantity of PCMB added and reaches a maximum in the presence of 14 equiv. of PCMB.

The addition of further amounts of PCMB has no longer any influence on the viscosity. After denaturation with 8 *M* urea we found the $[\eta]$value to be about 43.0.

Reversibility of the process

It is known that the PCMB inhibition of the enzymic activity of the PGAD can be abolished by the use of cysteine. Therefore the reversibility of the effect of the PCMB on the structure of the protein was studied. 15 equiv. of PCMB were added to the protein solution, and after incubating it at 20° for 1 to 15 min., 80 to 100 equiv. of cysteine were added, and the optical rotation was measured. The results are shown in Table I. It can be seen that during the first minutes, the PCMB effect is reversible. As the PCMB effect progresses in time, the reversibility ceases and after a longer period of time even somewhat higher, more negative values are measured, than without cysteine.

TABLE I

REVERSIBILITY OF THE EFFECT OF THE PCMB ON THE SPECIFIC OPTICAL ROTATION OF THE SWINE MUSCLE PGAD

Reagents added		*Time of incubation with PCMB (min)*	*Specific optical rotation* $[\alpha]_D^{20}$
PCMB (equiv.)	*cystein 80 to 100 µmole*		
None	None	—	— 33.3
15	None	15	— 60.2
15	+	1	— 37.9
15	+	10	— 58.9
15	+	18	— 68.3

DISCUSSION

According to these data, both the configuration and the characteristic hydrodynamic volume of the protein molecule undergo changes when the sulfhydryl groups of the PGAD are blocked with PCMB. All these changes cause conversions in the direction of denaturation. (a) In the presence of 14 to 15 equiv. of PCMB enzymic activity (*i.e.* oxidation of D-glyceraldehyde-3-phosphate) is reduced to zero[10]. (b) In the presence of 14 to 15 equiv. of PCMB the rate of digestion by trypsin reaches a maximum value which, however, is lower than that of the urea denatured PGAD[3]. (c) As compared to the native protein, optical rotation becomes significantly more negative in the presence of 14 to 15 equiv. of PCMB, as a result of an unfolding of part of the right handed helix structure. (d) In response to PCMB treatment the intrinsic viscosity *i.e.* the hydrodynamic volume of the protein increases also.

It is assumed that all these effects reach a maximum in the presence of 14 to 15 equiv. of PCMB, because then all the sulfhydryl groups in the protein are blocked[9]. The observations indicate that besides taking part in the enzymic reaction, (binding of substrate[11] and coenzyme[12, 13]), the sulfhydryl groups play a role of considerable importance in the stabilisation of the spatial configuration and in the right handed helix structure of the enzyme molecule. The changes resulting from blocking with PCMB vary in extent. Optical rotation increases in a relatively greater measure (from —30.7° to about —60°, maximum value after denaturation being —99.7°), than the intrinsic viscosity characterizing hydrodynamic volume (increase of $[\eta]$ from 3.2 to 8, maximum value after denaturation being about 43).

As to the reversibility of PCMB blocking, it is to be noted that in the tests on very dilute protein solutions a complete reversibility is observable in enzyme activity. Cysteine restores fully the activity of the completely blocked protein[13]. In our experiments the process became irreversible, when in the presence of higher protein concentration incubation with PCMB lasted longer than 20 min at 20°. Moreover, the results indicate that the process takes place in many phases: (a) PCMB links up with the protein, for a short time cysteine may help remove PCMB from the molecule. (b) As a result of the blocking of the sulfhydryl groups the secondary structure of the protein begins to disintegrate, the right handed helices unfold; this process is complete in 10 to 15 min in the presence of higher PCMB concentrations. (c) After part of the helix structure has disintegrated, groups become free on the surface of the protein, intermolecular linkages are formed and a precipitate begins to separate from the solution.

The mode in which the sulfhydryl groups—already studied—may stabilize the spatial structure and the configuration of the PGAD molecule requires a more extended study.

ACKNOWLEDGEMENTS

The author expresses his thanks to Mrs. E. Tóth for expert and skilful technical assistance and to Dr. G. Szabolcsi for valuable discussion.

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ON THE LOCALISATION OF OXIDASE SYSTEMS IN *ACETOBACTER* CELLS

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(Received July 28th, 1959)

SUMMARY

1. The oxidation of several carbohydrates and related compounds by intact cells of *Gluconobacter liquefaciens* has been studied.

2. Intact cells were disrupted by ultrasonic treatment and separated into several fractions by centrifugation. "Cell débris" and small particles oxidized glucose, gluconate and 2-ketogluconate to 2,5-diketogluconate and beyond; D-lactate and ethanol to the acetate stage; galactose, mannose, xylose and L-arabinose to the corresponding acids; sorbitol to sorbose; glycerol and *meso*-erythritol to the corresponding keto-derivatives. Acetate, 5-ketogluconate, fructose and mannitol were not attacked.

3. Intact cells could be converted into "protoplasts" with an efficiency of about 95 % by means of human serum in an appropriate medium. Pig serum effected 60–70 % conversion.

4. The "protoplasts" oxidized most of the substrates at about 60–80 % of the rate of the intact cells. The oxidation rate of substrates, metabolized after an induction period by intact cells (fructose, mannitol, 5-ketogluconate and glycerol) was considerably slower by "protoplasts".

5. Only about 50 % of the "protoplasts" lyzed in water or dilute buffer after several h at 30°. No lysis was observed after pig serum treatment.

6. Several fractions were obtained from "protoplasts" after either lysis or short ultrasonic treatment. All the substrates which were oxidized by the small particles and "cell débris", were likewise oxidized to the same extent by "ghosts" and "protoplast débris", with the exception of glycerol and *meso*-erythritol. The enzymes for these latter two substances were apparently inactivated at 37°.

7. Several particle-linked oxidases from *Gluconobacter* (*Acetobacter*) *suboxydans* were constitutive.

8. The oxidase-bearing particles from acetic acid bacteria apparently do not exist as such in the cytoplasm of the bacteria, but probably originate from "ghosts" through mechanical or ultrasonic disruption.

9. The oxidative characteristics, typical for the acetic acid bacteria, are mainly determined by the enzymic activity of an outer cell envelope (probably the cytoplasmic membrane).

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[*] Associé du Fonds National de la Recherche Scientifique (Belgique).

INTRODUCTION

The cells of acetic acid bacteria contain a variety of particle-linked enzymes, which carry out oxidations, typical for this genus. The particles from *Acetobacter suboxydans* are able to oxidize several sugars to the corresponding acids[1]. The particles, prepared from species of the mesoxydans and suboxydans groups, frequently also oxidize gluconate to 2-ketogluconate[1,2]. *Acetobacter peroxydans*[3] and many other species[4] contain particles oxidizing D- and L-lactate to acetate by way of pyruvate and acetaldehyde; ethanol and other primary alcohols are converted to the corresponding fatty acids. They also contain the complete system for electron transport from the substrate to O_2 by way of the cytochrome system. Particles from *A. peroxydans* carry out several peroxidatic reactions[5]. These ultramicroscopic structures were isolated in our laboratory from cell-free extracts by ultracentrifugation at 105,000 × *g* for 2 h. Other particulate fractions from *A. suboxydans*, usually isolated at lower speed and probably consisting of similar particles or aggregates therefrom, have been used by several authors[6-14]. These fractions were able to oxidize several cyclitols[6-9], several open chain polyols[10,11], glucose, galactose and primary alcohols[12]. They contained a δ-D-gluconolactone delactonase[13]. PRIEUR[14] discovered the particulate nature of the ethanol oxidase system in *A. xylinum*.

Although ultramicroscopic particles have been described in other bacterial genera (for a review, see ALEXANDER[15]), it appeared from the above mentioned studies that the *Acetobacter*-particles were rather unique because they displayed a far greater diversity of enzyme action. They were also very poor in RNA[2], which again set them apart from most of the particles from other bacteria.

In view of the presence of several oxidase systems, of the cytochromes[3,12] and of the low RNA content, we surmised that these particles were derived from the outer layer of the cell (most probably the cytoplasmic membrane[16,17]) and in the present paper experiments with intact cells, "protoplasts" and various subcellular fractions will be described which lend strength to this hypothesis.

EXPERIMENTAL METHODS

Bacteria used

The strain used was *Gluconobacter liquefaciens*, kindly supplied by PROF. T. ASAI, Tokyo. Several other strains from our collection were also used occasionally. The strain of *A. suboxydans* was the same as mentioned in a previous paper[1].

Growth conditions

The bacteria were inoculated in 10 ml of beer in 100 ml Erlenmeyer flasks and incubated for 2 days at 30° on a shaking machine. This suspension was used to inoculate the solid medium in Roux flasks, containing 10 % glucose, 1 % yeast extract (Difco), 3 % $CaCO_3$ and 2.5 % agar. For *A. suboxydans* the same medium with either 3 % galactose, 3 % xylose or 5 % mannitol as main carbon source was also used. The bacteria were harvested, centrifuged, washed twice in 0.01 *M* phosphate buffer pH 6.2 and finally suspended in the same buffer (100 mg living bacteria/ml). When the cells were to be used for "protoplast" production, they were suspended in 0.85 % NaCl solution.

Disruption of the cells (Fig. 1)

The cell suspension was treated in the 10 kc, 250-W Raytheon Sonic Oscillator for 15–20 min. Examination under the phase contrast microscope showed that nearly all the cells were broken. The opalescent suspension was centrifuged at 4° in the MSE centrifuge at 11,000 × *g* for 30 min to 1 h. The precipitate usually consisted of 3 layers: a bottom layer containing most of the intact cells, which was discarded, a distinctly brownish middle layer, called "brown layer", which was used occasionally and a white jellylike top layer ("white layer"). Since the enzymic activities of both layers were nearly identical, they were usually pooled and the fraction called "cell débris". The MSE supernatant was centrifuged in the Spinco Supercentrifuge at 105,000 × *g* for 2 h. The precipitate contained the brownish "particles A", equivalent to the particles which we studied in other strains. In ALEXANDER's nomenclature[15] they would be called the 105–120 fraction. The supernatant was called "supernatant A".

Manometric experiments were carried out in the conventional Warburg apparatus at 30°. Each vessel contained 50 mg of intact cells in a final volume of 2 ml 0.022 *M* phosphate buffer pH 6.2, with 10 µmoles of substrate in the side arm and 0.1 ml 15 % KOH in the central well. The oxidations were followed until cessation of O_2 consumption. In several cases where oxidation of substrates was slow, the process was followed up to 5 h.

Particles, "ghosts" and other fractions were suspended in 0.022 *M* phosphate buffer pH 6.2 and used in the same manner.

"Protoplasts" were centrifuged from the serum medium in which they were prepared and washed twice in 1 % $MgSO_4 \cdot 7H_2O$, 4 % lactose and 0.68 % NaCl, followed by centrifugation. For use in the Warburg they were suspended in the same medium which also contained 0.022 *M* phosphate buffer pH 6.2, with the substrate in the side arm. Final volume 2 ml. When the activity of the "protoplasts" was to be compared with that of the intact cells, the latter were suspended in the Warburg vessel in the same high-osmotic medium. Each vessel contained the amount of "protoplasts" prepared from 50 mg of intact cells. A small amount of intact cells, about 5 %, was still present and could not be separated by fractional centrifugation.

When the Spinco supernatant A or B was used, the oxidations were carried out with 0.65 mg *N*-methylphenaziniummethylsulfate per vessel as carrier.

RESULTS

1. Oxidations of several substrates by intact cells of Gluconobacter liquefaciens

Table I represents the results of a typical experiment.

Gluconate and 2-ketogluconate rapidly took up 1.0 and 0.5 O_2/mole substrate respectively, indicating the final formation of the same endproduct, 2,5-diketogluconate. The formation of this substance was also indicated by the fact that the content of the Warburg vessels turned brown after standing overnight. Furthermore, these cells produced a brown pigment when grown on a glucose medium; similarly *A. melanogenum*, is known to produce this pigment from 2,5-diketogluconate[18], Glucose took up rapidly about 2 O_2/mole substrate. Assuming that most of it was converted into 2,5-diketogluconate, one can calculate that about 10 % of the glucose was oxidized to completion, probably by way of the HMP oxidative cycle ("shunt"). The three above mentioned substrates were ultimately oxidized at exactly the same

TABLE I

THE OXIDATION OF SEVERAL SUBSTRATES BY INTACT CELLS AND "PROTOPLASTS" OF *Gluconobacter liquefaciens*

Manometric method at 30°; content of the Warburg vessels, see Methods. The oxidation rate is expressed as $Q_{\text{mole } O_2}$ (mole O_2 uptake/mole substrate/100 mg bacteria or "protoplasts"/h). The final O_2 uptake is expressed as mole O_2/mole substrate. When the reaction was slow and not yet over after 5 h, it was marked by the sign >.

Substrate	*Bacteria*		*"Protoplasts"*		$100 \times \frac{Q_{\text{mole } O_2}\text{ ("protoplasts")}}{Q_{\text{mole } O_2}\text{ (bacteria)}}$
	$Q_{\text{mole } O_2}$	*Final O_2 uptake*	$Q_{\text{mole } O_2}$	*Final O_2 uptake*	
Glucose	4.56	2.16*	3.00	2.2*	66
Sodium gluconate	5.70	0.97*	3.75	0.97*	66
Sodium 2-ketogluconate	5.06	0.50*	3.18	0.50*	63
Sodium D-lactate	5.29	2.85	4.28	2.85	81
Ethanol	6.06	2.63	4.66	2.63	77
Galactose	1.33	0.60	1.04	0.60	78
Mannose	0.34	>0.32	0.28	>0.40	83
Xylose	0.40	0.50	0.32	0.50	80
L-Arabinose	0.32	>0.31	0.28	>0.30	87
Sorbitol	0.40	0.40	0.26	>0.30	66
Glycerol	1.88	3.30	0.34	0.60	18
meso-Erythritol	0.60	0.80	0	0	0
Sodium acetate	1.18	2.10	0.71	>1.9	60
Sodium 5-ketogluconate	1.35	2.50	0.35	2.4	26
Fructose	1.58	>1.54	0.56	>0.8	35
Mannitol	1.54	>3.5	0.51	>1.2	34

* Followed by a slow further oxidation.

rate, which was apparently due to the further slow oxidation of 2,5-diketogluconate.

The following substrates were oxidized almost to completion: D-lactate, ethanol, acetate and glycerol. Both 5-ketogluconate and fructose were only partially oxidized (final O_2 uptake: 2.5 and 1.9 O_2/mole substrate respectively), suggesting either the formation of unidentified endproducts or a substantial assimilation. Mannitol was slowly oxidized, most probably also to near-completion. Sorbitol was oxidized to sorbose, which could be detected on paper chromatograms. Fructose was not formed. *Meso*-erythritol was oxidized slightly beyond the erythrulose stage, mannose, xylose and L-arabinose to the corresponding acids and galactose slightly beyond the galactonate stage.

Sorbose, D-arabinose, lactose and sucrose were not oxidized by these cells and ribose and maltose only very slightly or not at all.

It should be noted that 5-ketogluconate, glycerol, acetate, fructose and mannitol were only oxidized after an induction period of 10–30 min.

2. *Preparation of "protoplasts" from* Acetobacter

Several methods, described in the literature for the preparation of protoplasts, spheroplasts and similar structures (for a review, see WEIBULL[19]), have been used in our preliminary experiments for protoplast preparation from *Acetobacter*. The results were uniformly negative. With some methods, the conditions of pH, sucrose concentration or ionic strength were modified without improvement. Lysozyme treatment occasionally produced a few round forms. We are indebted to the Laboratory for

Hygiene, Prof. K. C. Winkler, Utrecht, for pointing out to us the serum method[20], which was discovered there by Dr. J. Willers, independently from the published method. Using this procedure, one strain, *Gl. liquefaciens*, was converted into approximately 95 % "protoplasts", as judged under the phase contrast microscope. 80 % "protoplasts" were obtained from *A. aceti muciparum* and 50 % from one strain of *A. suboxydans*. Most of the strains used, such as *A. mesoxydans, melanogenum, xylinum, peroxydans* and *Gl. cerinus* var. *rosei* were scarcely affected. It was found that species, consisting of small short cells, were unsuitable due to difficulty in observing differences between cells and "protoplasts". The term "protoplast" with quotation marks was used according to the proposal of Mitchell and Moyle[21] to denote the fact that the cell wall was not completely removed, in contrast to the real protoplasts from *Bacillus megaterium*.

The following modification of the method of Muschel *et al.*[20] was finally adopted. To 0.5 ml human or pig serum was added 0.1 ml 10 % $MgSO_4 \cdot 7H_2O$, 0.1 ml 40 % lactose and 10 mg of living bacteria in 0.3 ml 0.85 % NaCl. The suspension was incubated in a water bath at 37°, occasionally shaken and samples regularly observed under the phase contrast microscope. With human serum more than half of the cells were converted into spherical forms after 30 min. After 2–3 h there was about 95 % conversion. With pig serum, 5–12 h at 37° were required and only 60–70 % of the cells were converted. The intact cells were rods 3–4 or more μ long and about 1 μ thick. The "protoplasts" were perfect or nearly perfect spheres of about 1.5–2.5 μ diameter and often contained two or more granules in or on the membrane. Occasionally larger individuals were seen.

Influence of serum. Fresh human blood was obtained from the Dept. of Internal Medicine through the courtesy of Dr. A. Vermeulen. Serum was prepared as usual and centrifuged free of erythrocytes. It could be stored for several weeks at — 20° without loss of activity. There was no difference in the rate or final amount of "protoplasts" when serum was used from 3 people, currently working in this laboratory with *Acetobacter*, and from a variety of other people, who had never been exposed to *Acetobacter* cultures. However, with the latter types of serum there was usually a marked agglutination of the bacteria. Horse and cow serum agglutinated the bacteria rapidly and did not produce "protoplasts". 0.5–0.4 ml human serum/ml mixture was required, smaller amounts gave smaller yields. Substitution of 100 μg crystalline lysozyme for serum did not result in "protoplast" formation.

Influence of Mg^{++}. Omission of Mg^{++} scarcely affected the final yield of "protoplasts". On the whole the spheres were much smaller.

Influence of the stabilizer. 5 % sucrose, 4 % lactose or 5 % maltose could be used without notable difference. Omission of the stabilizer still resulted in a yield of 95 % "protoplast"; which were larger than usual. Lactose was preferred to sucrose, since *Gl. liquefaciens* was unable to oxidize it.

Mass production of "protoplasts". For physiological studies on the "protoplasts" and their subunits, several grams of a 2 day old culture of the bacteria could easily be converted in the conditions described above. The production of "protoplasts" was limited only by the amount of human serum available.

Lysis of the "protoplasts". The "protoplasts" were collected by centrifugation, washed twice in a solution containing 1 % $MgSO_4 \cdot 7H_2O$, 4 % lactose and 0.68 % NaCl. In the latter solution they were stable for at least 1 day. They were suspended in either distilled water or 0.01 M phosphate buffer pH 6.2 and kept at 30°; the lysis, which was slow, was followed by phase contrast. They did not burst as is the case for protoplasts of Gram-positive micro-organisms. Only after 3–4 h were an appreciable amount of "ghosts" noted. However, lysis was never complete, about 50 % of the "protoplasts" remaining unchanged. The "ghosts" had approximately the same dimensions as the original "protoplasts" and often retained the spherical shape, although it could be seen that they were empty and were no longer intact. They were nearly transparent and often the granules still adhered to them. It was found to be advantageous to prepare the "ghosts" immediately after the "protoplasts" were fully formed. Lysis was only obtained from "protoplasts" made with human serum; with pig serum scarcely any lysis was noted. After lysis the suspension was separated still further as described in Fig. 1.

Disruption of the "protoplasts". The "protoplasts" were also disrupted by controlled breakage in the Raytheon Sonic Oscillator. Every min a sample was taken and observed under the phase contrast microscope. 4–7 min treatment broke the "protoplasts" almost completely, as compared to the 20 min required for intact cells. The

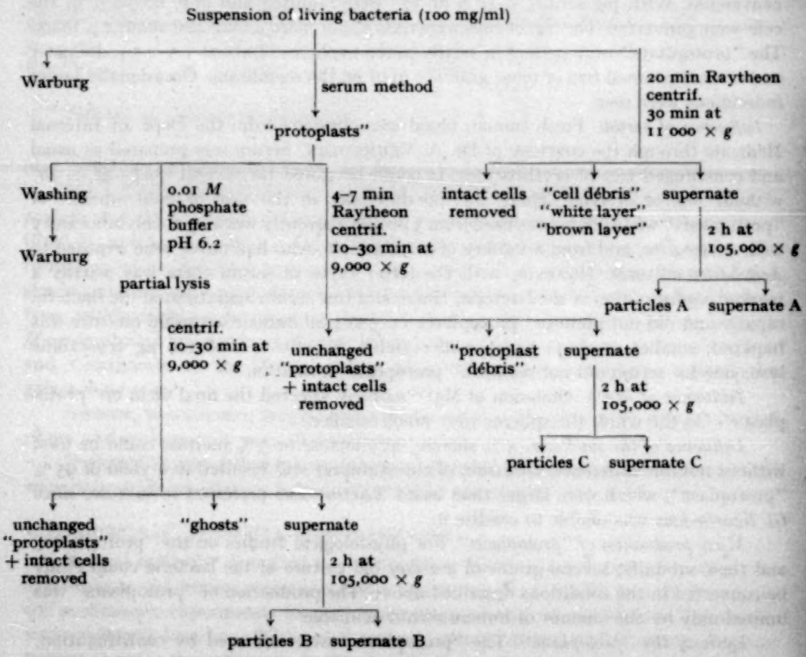
Suspension of living bacteria (100 mg/ml)
Warburg
serum method
20 min Raytheon
centrif.
30 min at
11 000 × g
"protoplasts"
Washing
Warburg
0.01 M
phosphate
buffer
pH 6.2
partial lysis
4–7 min
Raytheon
centrif.
10–30 min at
9,000 × g
intact cells
removed
"cell débris"
"white layer"
"brown layer"
supernate
2 h at
105,000 × g
particles A
supernate A
centrif.
10–30 min at
9,000 × g
unchanged
"protoplasts"
+ intact cells
removed
"protoplast
débris"
supernate
2 h at
105,000 × g
particles C
supernate C
unchanged
"protoplasts"
+ intact cells
removed
"ghosts"
supernate
2 h at
105,000 × g
particles B
supernate B

Fig. 1. Flow-sheet of the preparation of "protoplasts" and various subcellular fractions from *Gluconobacter liquefaciens*.

results of the fractional centrifugation can be seen from Fig. 1. The upper layer was called "protoplast débris". Under phase contrast this fraction contained small fragments about 1 μ or less in diameter and many membraneous "ghosts" or "ghost fragments". The amount of particles B and C, after centrifugation of the supernatant in the Spinco supercentrifuge, was very small.

3. Oxidation of several substrates by "protoplasts" of Gl. liquefaciens (Table I, Fig. 2)

The "protoplasts" were still able to actively oxidize most substrates. The ten upper substrates, listed in the Table I, were oxidized at a slightly decreased rate as compared to intact cells. This decrease was probably due to either a partial inactivation of the "protoplasts" which were kept at 37° for several h, or to the destruction of some of them. We shall show below that the oxidation of all these substrates was also effected by "ghosts" and particle-linked enzymes. Acetate was oxidized after a marked induction period. In spite of the relatively high respiration rate acetate really belonged to the following group of substrates.

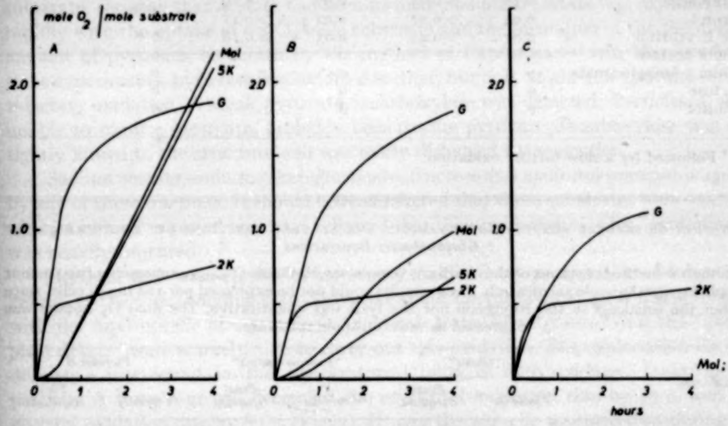

Fig. 2. Oxidation of glucose (G), mannitol (Mol), 5-ketogluconate (5K) and 2-ketogluconate (2K) by intact cells (Fig. A), "protoplasts" (Fig. B) and "ghosts" (Fig. C) of *Gl. liquefaciens*. Manometric method, content of the Warburg vessels, see text.

The oxidation rate of 5-ketogluconate, fructose, mannitol, glycerol and *meso*-erythritol was lowered for 60–100 %, depending on the substrate. The oxidation curve of the latter substrates (including acetate but excluding *meso*-erythritol), showed a more or less marked induction period. These results suggest that the latter substrates were oxidized by way of one or more inducible enzymes and that the capacity of the "protoplasts" to form those enzymes was seriously impaired. It is very striking that all these enzymes (kinases and coenzyme-linked dehydrogenases) were soluble and that these substrates were oxidized neither by the "ghosts", the particles nor any other fraction.

4. Oxidation of several substrates by subcellular fractions of Gl. liquefaciens (Tables II and III)

TABLE II

OXIDATION OF SUBSTRATES BY SEVERAL FRACTIONS OF RAYTHEONIZED INTACT CELLS OF *Gluconobacter liquefaciens*

Manometric method; content of the Warburg vessels, see Methods. The results are expressed as in Table I.

Substrate	*Particles A*		*"Cell débris"*		*Spinco Supernatant A*	
	$Q_{\text{mole } O_2}$	*Final O_2 uptake*	$Q_{\text{mole } O_2}$	*Final uptake O_2*	$Q_{\text{mole } O_2}$	*Final O_2 uptake*
Glucose	3.42	1.1*	1.04	>1.3*	0.12	>0.3
Sodium gluconate	3.28	0.95*	1.40	0.95*	0.30	>1.0
Sodium 2-ketogluconate	2.60	0.5*	1.42	0.5*	0.18	>0.8
Sodium D-lactate	1.68	0.5	0.58	0.5	0	
Ethanol	2.73	0.95	1.41	0.97	0	
Galactose	0.21	0.5	0.14	>0.4	0	
Mannose	0.05	>0.1			0	
Xylose	0.11	>0.35	0.06	>0.26	0	
L-Arabinose	0.08	>0.35	0.07	>0.19		
Glycerol	0.05	>0.28	0.02	>0.11		
meso-Erythritol	0.07	>0.45	0.03	>0.18	0	
Sodium acetate	0		0		0	
Sodium 5-ketogluconate	0		0		0	
Fructose	0		0		0	
Mannitol	0		0		0	

* Followed by a slow further oxidation.

TABLE III

OXIDATION OF SEVERAL SUBSTRATES BY VARIOUS SUBCELLULAR FRACTIONS OF "PROTOPLASTS" OF *Gluconobacter liquefaciens*

Manometric method; content of the Warburg vessels, see Methods. $Q'_{\text{mole } O_2}$ represents the amount of mole O_2 uptake/mole substrate/h. These results could not be expressed per 100 mg of cells, since neither the breakage in the Raytheon nor the lysis was quantitative. The final O_2 uptake was expressed as mole O_2/mole substrate.

Substrate	*"Ghosts"*		*"Protoplast débris"*		*Particles B*	
	$Q'_{\text{mole } O_2}$	*Final O_2 uptake*	$Q'_{\text{mole } O_2}$	*Final O_2 uptake*	$Q'_{\text{mole } O_2}$	*Final O_2 uptake*
Glucose	0.88	>1.1	0.50	>1.0	0.6	<1.0
Sodium gluconate	0.36	0.9	0.16	>0.6		
Sodium 2-ketogluconate	0.36	0.5	0.28	0.6		
Sodium D-lactate	0.60	0.6	0.13	>0.9		
Ethanol	0.15	>0.7	0.10	>0.6	0.07	1.0
Galactose	0.07	0.5	0.10	0.5	0	
Mannose	0.03	>0.25				
Xylose	0.05	>0.35	0.08	>0.32	0	
L-Arabinose	0.04	>0.30				
Glycerol	0		0		0	
meso-Erythritol	0		0		0	
Sodium acetate	0		0		0	
Sodium 5-ketogluconate	0		0		0	
Fructose	0		0		0	
Mannitol	0		0		0	

The behaviour of the "ghosts", "protoplast débris", "cell débris" and particles A will be discussed together, because their oxidative capacities were nearly identical.

2-Ketogluconate was always oxidized with a rapid uptake of 0.5 mole O_2/mole substrate, with the formation of 2,5-diketogluconate followed by a slow oxidation of the latter substance. Only the "white layer" appeared to lack the latter oxidase. The oxidation of 2-ketogluconate to 2,5-diketogluconate was also in *A. melanogenum* almost certainly carried out by particles, since KATZNELSON *et al.*[18] reported that 0.5 O_2 was taken up with crude extract, in the absence of an artificial electron carrier. Gluconate took up rapidly about 1.0 and glucose 1.1–1.3 mole O_2/mole substrate. For both substrates the oxidation rate continued slowly at the same rate as the 2,5-diketogluconate oxidation.

Galactose, xylose and L-arabinose were always oxidized with a final uptake of 0.5 mole O_2/mole substrate. The oxidation of mannose was much slower, but tended to the same value. It was obvious that in all these cases the corresponding sugar acids were formed. Sorbitol was very slowly oxidized by particles A and, as a consequence, this substrate was not investigated further.

All these fractions oxidized ethanol with the final uptake of 0.9–1.0 mole O_2/mole substrate, showing that acetate had been formed. Sodium D-lactate was oxidized very rapidly with the uptake of 0.5 O_2/mole substrate and the formation of the theoretical amount of pyruvate, as shown by the method of FRIEDEMANN AND HAUGEN[22]. In some experiments, pyruvate was oxidized further, but only at about 6 % of the rate of D-lactate oxidation. A weak pyruvate decarboxylase was detected. Particles A were unable to oxidize pyruvate, probably because the pyruvate decarboxylase was not tightly linked to the structure and was easily dislodged by sonication.

Sodium acetate, sodium 5-ketogluconate, fructose and mannitol were not oxizided by any of these fractions. It should be remembered that these substrates were oxidized after an induction period by intact cells and that their oxidation rate by "protoplasts" was heavily impaired.

Glycerol and *meso*-erythritol were only oxidized by particles A and by "cell débris" to the corresponding keto-derivative stage, dihydroxyacetone and erythrulose, with the final uptake of 0.5 mole O_2/mole substrate. The "ghosts" and the "protoplast débris" were scarcely able to carry out this oxidation. The explanation for this difference was found in the temperature-lability of both oxidases. Treatment of particles A for 3 h at 37° decreased the erythritol oxidation rate by 75 % and the glycerol oxidation rate by 60 % (Fig. 3). It may therefore be assumed that during the preparation of the "protoplasts" at 37°, both oxidase systems, which were probably located on the "ghosts", were almost completely destroyed.

When the "ghosts" were treated in the Raytheon for 20 min, they could no longer be centrifuged at 9,000 × *g* for 30 min — 1 h. After centrifugation of this suspension at 105,000 × *g* for 2 h a precipitate was formed which was indistinguishable from the particles A.

The Spinco supernatants A and B were only able to oxidize glucose, gluconate and 2-ketogluconate weakly in the presence of phenazine. The other substrates were not attacked, showing that indeed most of the oxidase systems were nearly always located on the above mentioned insoluble structures.

The amount of particles B and C being very small, only a few experiments could be carried out. The results indicated however that their enzymic activity was nearly

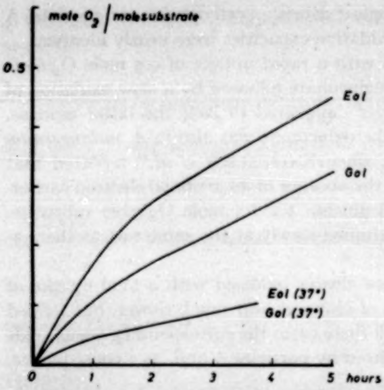

Fig. 3. Influence of the temperature on the stability of the oxidase systems for glycerol (Gol) and *meso*-erythritol (Eol) in particles A from *Gl. liquefaciens*. Manometric method at 30°. Content of the Warburg vessels, see text. The particles, used for the curves Gol and Eol, were not exposed to temperatures higher than 4° before the manometric experiment. For the curve with index 37°, a batch of particles was used, kept at 37° for 3 h prior to the experiment.

negligible. These fractions may correspond to the RNA-containing particles isolated by MARR *et al.*[23,24] and require further study.

5. *The constitutive nature of particle-linked enzymes*

Some of the above experiments suggested that the particle linked enzymes were constitutive. This was tested by the following experiments. *A. suboxydans* was grown in Roux flasks on a solid medium described above with either 10 % glucose, 3 % galactose, 3 % xylose or 5 % mannitol as main carbon source. The cells were grown for 2 days at 30°, harvested, washed, broken in the Raytheon for 15 min and particles A isolated as described for *Gl. liquefaciens*. Particles from these four batches of cells behaved in an identical fashion. Glucose and gluconate were oxidized to 2-ketogluconate (demonstrated by paper chromatography), galactose, L-arabinose and xylose to the corresponding sugar acids. Mannitol was oxidized to fructose (identified by paper chromatography). Glycerol, inositol, *meso*-erythritol, sorbitol and adonitol were oxidized with the uptake of 0.5 O$_2$/mole substrate. Mannose, maltose and sucrose were slowly oxidized. A variety of other sugars and sugar derivatives were not attacked. This experiment showed that the particles contained the same oxidase systems, irrespective of the conditions in which the cells had previously been grown.

A further argument was derived from the oxidation rates by these particles. They oxidized the above mentioned substrates at practically the same relative rate (glucose arbitrarily taken as 100) (Table IV).

DISCUSSION

It seems very likely that the cell wall of *Acetobacter* species may be of a rather unusual constitution or structure, since it could not be removed by any of the methods now in use for the preparation of the protoplasts. The serum method alone was successful and then only with a limited number of strains. Serum undoubtedly removed part of the cell wall of the intact cells, since spherical bodies were obtained with fragile membranes. The latter fact could be readily observed by ultrasonic vibration. Intact

TABLE IV

THE OXIDATION RATE OF SOME SUBSTRATES BY PARTICLES, ISOLATED FROM *A. suboxydans*, GROWN ON SEVERAL SUBSTRATES

Content of the Warburg vessels (30°) in 2 ml 0.022 *M* phosphate buffer pH 6.2: particles (final turbidity 240 as measured in the Klett colorimeter with filter 66), 10 μmoles of substrate in the side-arm. 0.1 ml 15% KOH in the central well. The oxidation rate of glucose was always arbitrarily set as 100.

Substrate	*Particles from bacteria grown on*			
	glucose	*galactose*	*xylose*	*mannitol*
Glucose	100	100	100	100
Sodium gluconate	50	40	76	38
Galactose	11	13	11	8
L-Arabinose	15			10
Xylose	28	22	25	37
Mannitol	15			11
Inositol	145			152
meso-Erythritol	31			34

cells required 15–20 min of sonication for nearly complete breakage, while "protoplasts" were disrupted after 4–7 min.

For the slow lysis of the "protoplasts" there are several explanations. The hypothesis that the "protoplast" membrane retained a certain rigidity and was not as delicate and fragile as the "ghost" of Gram-positive organisms seems the most likely because of the large swollen "protoplasts" which were often formed after several h in distilled water. The *Acetobacter* "protoplasts" resembled the spheroplasts and similar structures, described in other Gram-negative micro-organisms[19].

The "ghosts", the "cell débris", the "protoplast débris" and the particles A all oxidized the same substrates with the formation of the same end products. The oxydation of glycerol and *meso*-erythritol was the only exception, apparently because heat-labile enzymes were involved. These results thus tend to show that the oxidase bearing particles were derived from the cell envelope (probably the cytoplasmic membrane) through ultrasonic breakage. An alternative explanation might be *e.g.* that these particles tightly adhere to the cytoplasmic membrane and are dislodged by ultrasonic treatment. Anyway, these results strengthen the view that these particles do not float freely in the cytoplasm and that they may not even exist at all as separate structures in the cell. MARR *et al.*[23, 24] have shown that the small particles of *Azotobacter vinelandii*, bearing hydrogenase and several oxidase systems, were artificially derived from the "hull" through sonication. The latter also displayed the same enzymic activity as the particles.

The "ghosts" of *Acetobacter* cells have enzymes for the oxidation of several hexoses, pentoses, gluconate, 2-ketogluconate in the case of *Gl. liquefaciens* and possibly *A. melanogenum*, D-lactate, ethanol, several sugar alcohols and the complete electron-transport system, with the formation of the corresponding hexonic and pentonic acids, 2-ketogluconate, 2,5-diketogluconate for the 2 mentioned species, acetate and ketosugars. It is likely, but still has to be proved, that *all* the particle-linked enzymes in the genus *Acetobacter*[1–14], are really "ghost"-linked. This would considerably simplify the picture of the structure and the physiological activities of *Acetobacter* cells. The main characteristics of acetic acid bacteria reside precisely in these highly developed oxidative capacities, which set them apart from most other bacterial genera. This then would be mainly a reflection of the special structure and enzymic activity of their cell envelope (cytoplasmic membrane). Furthermore, it is worth stressing that these membrane-linked enzymes appear to be constitutive. This picture of the anatomy of the *Acetobacter* cell coincides with the results of other authors on the localisation of several enzymes in the plasma membrane of *Staphylococcus aureus*[16], the "hull" of *Azotobacter vinelandii*[23, 24] and the "ghost" in *Bacillus megaterium*[17].

Finally, a remark on the taxonomic and phylogenetic position of *Gl. liquefaciens* is desirable. Leifson[25] proposed to reassemble the polarly flagellate acetic acid bacteria in a new genus *Acetomonas*, *e.g. A. suboxydans* and *A. melanogenum*. Asai has recently stressed[26] that he segregated a group of acetic acid bacteria in a new genus *Gluconobacter*[27], at that time based on several physiological properties. Both *Acetomonas* and *Gluconobacter* are obviously identical and it appears to us that, for priority reasons, the latter name should be retained. The inability to oxidize acetate to CO_2 and H_2O is one of the characteristics of this genus. From the above experiments, from similar results of Stouthamer[28] and from the behaviour of growing cultures[26], it may be seen that *Gl. liquefaciens* is able to oxidize acetate. Yet, it leaves little doubt that this species belongs to the genus *Gluconobacter* (and is not a member of the mesoxydans group, as erroneously stated[28]), because of the type of flagellation, the strong gluconic acid production, the sugar alcohol oxidation and the γ-pyrone formation[26, 27]. *Gl. liquefaciens* has the most extensive enzymic array of all acetic acid bacteria described so far and one can imagine that, in the course of evolution, it could have given rise to *Gl. melanogenum* by loss of the Krebs cycle. Further loss of the 2-ketoglucono-oxidase would then lead to *Gl. suboxydans* and *Gl. cerinus*.

ACKNOWLEDGEMENTS

One of us (J. D. L.) is indebted to the Nationaal Fonds voor Wetenschappelijk Onderzoek for a grant and to Miss D. Lesaffre for skilful technical assistance.

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THE SYNTHESIS AND METABOLISM OF PROGESTERONE IN THE HUMAN AND BOVINE OVARY

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(Received September 8th, 1959)

SUMMARY

1. The biosynthesis of progesterone from [1-^{14}C]acetate has been demonstrated.

2. By means of *in vitro* incubations of [4-^{14}C]progesterone and [4-^{14}C]androstenedione in human ovarian tissue, a continuous spectrum of intermediate compounds leading to the synthesis of estrogens has been demonstrated. Presumptive evidence has been obtained for the following products: 6β-hydroxyprogesterone, pregnanedione, allopregnanedione, 20α-hydroxypregnene-3-one, 20β-hydroxypregnene-3-one, 17-hydroxyprogesterone, 17α-20ξ-dihydroxypregnene-3-one, 20ξ-hydroxypregnane-3-one, androstenedione, estrone, and estradiol-17β.

INTRODUCTION

Although it has been assumed that the early steps of progesterone biosynthesis follow the same general pathway as the synthesis of cholesterol[1,2], direct evidence for its synthesis from simple carbon compounds in the ovary has not been heretofore available. The studies reported in this paper demonstrate the *in vitro* biosynthesis of progesterone from [1-^{14}C]acetate incubated with corpora luteal tissue. The subsequent course of progesterone metabolism in the human ovary has been followed by the *in vitro* incubation of [4-^{14}C]progesterone and [4-^{14}C]androstenedione with ovarian stromal and corpora luteal tissue. Seven products have been characterized. The nature of the products is such as to suggest a relatively detailed metabolic scheme in the transformation of progesterone to androstenedione and estrogens.

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* Key investigator, American Cancer Society.

Biochim. Biophys. Acta, 40 (1960) 289–296

EXPERIMENTAL

Minced human stromal or luteal tissue[1] (0.5–1.0 g) or bovine corpora luteal tissue (5–6 g) was incubated with chromatographically pure radioactive compounds (human tissue: 10,000 counts/min-[4-^{14}C]progesterone, specific activity $1.07 \cdot 10^7$ counts/min/mg; 10,000 counts/min-[4-^{14}C]androstenedione, specific activity $1.11 \cdot 10^7$ counts/min/mg; 10,000 counts/min-[4-^{14}C]testosterone, specific activity $1.1 \cdot 10^7$ counts/min/mg—bovine tissue: 1,000,000 counts/min-[1-^{14}C]sodium acetate, specific activity 30.5 μC/mg) in a buffer medium (0.05 *M* sodium–potassium phosphate, pH 7.3 and 0.004*M* magnesium chloride) at 37° for 2 h (bovine tissue, 4 h). The incubations were terminated by addition of 3 volumes of acetone which was subsequently removed by vacuum distillation. The residue from a chloroform extract of the water solution was dissolved in a few drops of chloroform–methanol (1 : 1) mixture and chromatographed in ZAFFARONI's[3] formamide systems. The radioactive areas were located by means of an automatic strip feed counter[4] and analyzed by methods heretofore described[5].

Preparation of [4-^{14}C]androstenedione

This radioactive compound was prepared by dissolving 500,000 counts/min of testosterone in 15 drops of glacial acetic acid to which one small dry crystal of chromium trioxide was added. The mixture was shaken thoroughly in a glass stoppered tube and allowed to stand overnight at room temperature. The following morning 7 ml of water were added to the mixture, immediately after which the steroid was extracted with ethyl acetate. The ethyl acetate was removed with a stream of nitrogen and the residue was dissolved in methanol–chloroform (1 : 1) for chromatography on the hexane–benzene system. The androstenedione area was eluted from the paper with methanol and dried for use in the experiment.

ZIMMERMAN *reaction*[6] *for detection of ketonic carrier compounds*

A thin longitudinal strip of the chromatogram was dipped in a mixture of two volumes of *m*-dinitrobenzene (1 % in methanol) and one volume of freshly prepared 15 % potassium hydroxide and dried on a medium warm glass plate, to develop the color. (3 keto compounds gave a blue violet color; 17 keto compounds gave a violet color and 20 keto groups gave a brown purple color.)

Ferric chloride test[7] *for detection of carrier estrogens*

A longitudinal strip or sample of the chromatogram was dipped in a mixture consisting of equal volumes of 1 % ferric chloride ($FeCl_3$) and 1 % $K_3Fe(CN)_6$ and subsequently washed in 0.1 *N* hydrochloric acid. Excess hydrochloric acid was removed by washing in tap water and the chromatogram was allowed to dry.

Reduction of ketonic groups

2 ml of a saturated solution of $LiAlH_4$ in anhydrous diethyl ether were added to the dry steroid, mixed thoroughly and allowed to stand at room temperature for 5 min. 3–4 ml of distilled water was then added to stop the reaction and decompose the excess $LiAlH_4$. The reduced steroid was then extracted from the solution with ethyl acetate which was removed by evaporation under a stream of nitrogen.

20α-and 20β-hydroxypregnene-3-one were generously supplied by Dr. W. KLYNE

(Post Graduate Medical School, London). 6β-hydroxyprogesterone was a gift from Syntex, S.A.. The other steroids were obtained from commercial sources.

RESULTS

Biosynthesis of progesterone

Fig. 1 is a tracing of the radiochromatograph obtained from an incubation of bovine corpora luteal tissue with [2-^{14}C]acetate.

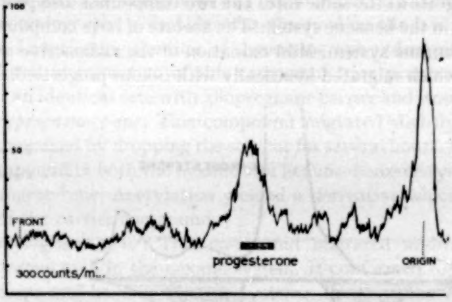

Fig. 1. Biosynthesis (bovine corpora luteal tissue) of progesterone from [1-^{14}C]acetate. The crosshatched area represents non-radioactive (carrier) progesterone (detected by u.v. absorption).

The peak at the mid-portion of the chromatogram was eluted and rechromatographed in the hexane–formamide and heptane–formamide systems with carrier progesterone. In both systems the radioactive peak migrated at the same rate as the carrier compound. The specific activities of the two chromatograms from two solvent systems were shown to be the same (hexane–formamide, 2.04 counts/min/mg; heptane–formamide, 2.06 counts/min/mg. 93 and 85 μg respectively of the 100 μg progesterone originally added were recovered from the chromatogram). The compound was shown to be not acetylatable. Three consecutive crystallizations of the mixture of progesterone and the unknown radioactive compound from two solvents (heptane and hexane) exhibited the same specific activities:

	Crystallization	*Specific activity*
1)	(Hexane)	53.0 counts/min/mg
2)	(Heptane)	57.1 counts/min/mg
3)	(Heptane)	56.3 counts/min/mg

Identification of the other radioactive peaks was not carried out in these experiments.

Metabolism of progesterone

The chromatographic spectra of progesterone metabolism in the human ovary are shown in Fig. 2. The initial spectrum is a tracing of a radio-chromatograph obtained after the incubation of 10,000 counts/min [4-^{14}C]progesterone in 0.5 g of human corpora luteal tissue. The subsequent spectra were obtained from composite extracts from incubations of both stromal and corpora luteal tissue. Essentially the same compounds have been found in both types of tissue. The figure also shows the sequence in which the various radioactive areas were resolved for analysis. The following is presumptive evidence for the identity of the isolated compounds:

1. *6β-hydroxyprogesterone*: The radioactivity remaining at the origin in the original hexane–formamide chromatogram was rechromatographed in the hexane–benzene system with the carrier compound (non-radioactive 6β-hydroxyprogesterone) and found to migrate at the same rate. The two compounds also positioned themselves in the same area in the benzene system. The acetate of both compounds ran identically in the hexane–benzene system. Mild oxidation of the radioactive compound resulted in a derivative which migrated identically with 6-keto-progesterone.

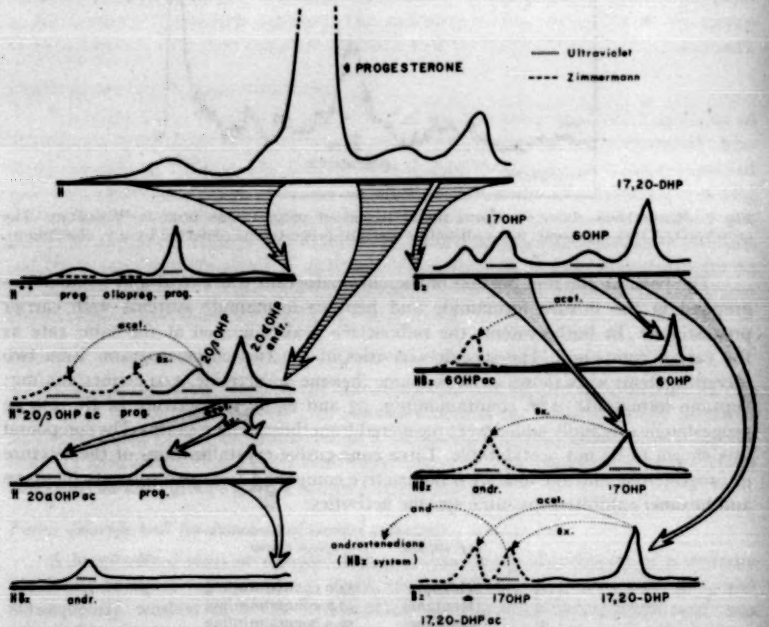

Fig. 2. Chromatographic spectra of progesterone products in the human ovary and the sequence of analysis. The wide portions of the hatched arrows represent the area of the chromatogram eluted. The arrows point to subsequent treatment of the eluted radioactive material. The following abbreviations are employed: H, chromatography to front in hexane system; H*, chromatography in hexane dropped 1 h from front; H**, chromatography in hexane dropped 1–1.5 h from front; Bz, chromatography to front in benzene system; HBz, chromatography to front in hexane–benzene system; prog., progesterone; preg., pregnanedione; allopreg., allopregnanedione; 20α-OHP, 20α-hydroxypregnene-3-one; 20βOHP, 20β-hydroxypregnene-3-one; 6βHP, 6β-hydroxyprogesterone; 17OHP, 17α-hydroxyprogesterone; 17,20-DHP, 17α, 20ξ-dihydroxypregnene-3-one; andr., androstenedione; ac, acetate ester; ox, oxidation; acet., acetylation. The dotted lines represent areas in which the carrier compounds were detected with either the Zimmermann reaction or u.v. absorption. A carrier compound was not available for the 17, 20-DHP compound.

2. *Pregnanedione*: Initial and secondary chromatography in the hexane system proved that the radioactive compound migrates identically with the index carrier (R_F 0.6–0.8). Reduction of the combined compounds gave a more high polar compound (probably monohydroxy compounds, as they were of intermediate polarity) which ran identically with the reduced carrier. Upon acetylation of the reduced compounds (carrier and radioactive), the radioactive and non-radioactive compounds were found in the identical area of the chromatogram. The original compound did not form an acetate ester.

3. *Allopregnanedione*: This compound was not found in all incubations. However, in some of the chromatographs a shoulder was observed on the low polar side of the progesterone peak. Lengthening the time of chromatography (dropping of the solvent for one hour) resulted in resolution of this compound from progesterone. The compound migrated at an identical rate with allopregnane carrier and would not acetylate.

4. *20α-hydroxypregnene-3-one*: This compound migrated slightly from the origin in hexane and was resolved by dropping the solvent for several hours. It ran identically with the index compound in both the hexane and hexane–benzene systems. Oxidation converted it to progesterone. Acetylation yielded a derivative which ran identically with the acetate of the carrier compound.

5. *20β-hydroxypregnene-3-one*: This compound migrated midway between the origin and progesterone area in the hexane system. It conformed to the carrier compound in both hexane and hexane–benzene systems. Progesterone was formed upon oxidation. When the combined area containing the index compound and the radioactive compound was acetylated both migrated to the front of the hexane chromatographic system.

6. *17-hydroxyprogesterone*: This compound had an R_F of approx. 0.3 in hexane–benzene and 0.6 in the benzene systems. In both systems it ran identically with the index compound. Oxidation converted it to androstenedione. It did not form an acetate ester.

7. *17α–20ξ-dihydroxypregnene-3-one*: In benzene the radioactivity corresponding to this compound exhibited an R_F of 0.25; in chloroform, an R_F of 0.6. Its acetate migrated between the front and 17-hydroxyprogesterone in the benzene system (R_F 0.9). The acetate had an R_F in the hexane–benzene system of 0.5. Mild oxidation gave as products, 17-hydroxyprogesterone and androstenedione. The observations that this compound forms an acetate which migrates at a slightly faster rate than 17-hydroxyprogesterone, and yields 17-hydroxyprogesterone and androstenedione upon mild oxidation is strong evidence for the structure listed. As 20α-hydroxypregnene-3-one is the major product in ovarian progesterone metabolism, it is likely that the compound is 17α,20α-dihydroxypregnene-3-one.

8. *20ξ-hydroxypregnane-3-one*: The R_F of this compound in the hexane–formamide system is almost identical to that of progesterone. Upon acetylation of the progesterone peak, two products are noted: Unchanged progesterone and an acetylated compound which migrates to the front. When the compound is hydrolyzed to remove the acetate group and subsequently oxidized (mild), a derivative is observed which migrates at the same rate as pregnanedione. As the reduction of the 20 ketone to a 20α-hydroxy group seems to dominate in the human ovary, this compound is thought to be 20α-hydroxypregnane-3-one.

9. *Androstenedione*: In the initial chromatography with hexane, this compound

migrates slightly in front of the origin. In the hexane–benzene system it has an R_F of 0.65. It migrates identically in both systems with the index compound and does not form an acetate.

Only small radioactive areas corresponding to the following compounds were observed in chromatographs from the progesterone incubations. However, when [4-^{14}C]androstenedione was employed as the substrate, radioactive areas of sufficient magnitude to acetylate and rechromatograph (Fig. 3) were obtained.

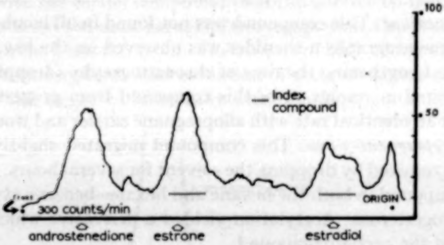

Fig. 3. Tracing of a radiochromatograph demonstrating the synthesis of estradiol and estrone from [4-^{14}C]androstenedione (ovarian stromal or luteal tissue).

10. *Estrone*: In both the hexane–benzene and benzene systems the radioactive compound migrates identically with index estrone. The acetylated derivative has an R_F of about 0.65 in hexane–formamide and migrates with estrone acetate.

11. *Estradiol-17β*: The radioactivity of this compound migrates at the same rate as index estradiol-17β chromatographed in the benzene and chloroform systems. Acetylation forms an acetate which runs with the front in hexane.

12. *Origin–peak* (Fig. 3): The radioactive compound in this peak has not been studied.

DISCUSSION

Fig. 4 depicts possible steps in the conversion of progesterone to androstenedione and the estrogens. All products listed in the figure which precede the step of aromatization can be readily demonstrated in a continuous spectrum after [4-^{14}C]progesterone is incubated in human corpora luteal or stromal tissue. Only two of the products, 20α-hydroxypregnene-3-one and 20β-hydroxypregnene-3-one have been heretofore isolated from normal human ovaries[8]. Both 17-hydroxyprogesterone and androstenedione have been reported as products of progesterone metabolism in tissue from an arrhenoblastoma[9] and polycystic (Stein-Leventhal) ovaries[10]. The latter investigators[10] also reported tentative identification of testosterone, but several studies[11] with polycystic ovaries in this laboratory have revealed only those products listed in Fig. 4.

In the current experiments only traces of estrone and estrodiol have been observed after incubation with [4-^{14}C]progesterone. However, the formation of both estrone and estradiol were readily demonstrated when [4-^{14}C]androstenedione was incubated as a precursor (Fig. 3). The compound/s remaining at the origin (Fig. 3) has not been identified. However, its failure to form an acetate ester indicates it is not estriol.

The metabolism of progesterone in the human ovary seems similar to that in experiments. HEARD *et al.*[18] and WEST *et al.*[19] have demonstrated the *in vivo* conversion of testosterone to estrogens. It has been shown in this investigation as well as in investigations by others[20, 21] that testosterone can be converted to estrogens in the human ovary. Placental tissue also can carry out this conversion[22] as well as other conversions[23]. The present experiments suggest that androstenedione is the dominating precursor in the course of synthesis of the estrogens. The synthesis of both estrone and estradiol can be demonstrated from this precursor (Fig. 3).

The significance of the formation of pregnanedione, allopregnanedione, 6β-hydroxyprogesterone and 20ξ-hydroxypregnane-3-one (latter compound not included

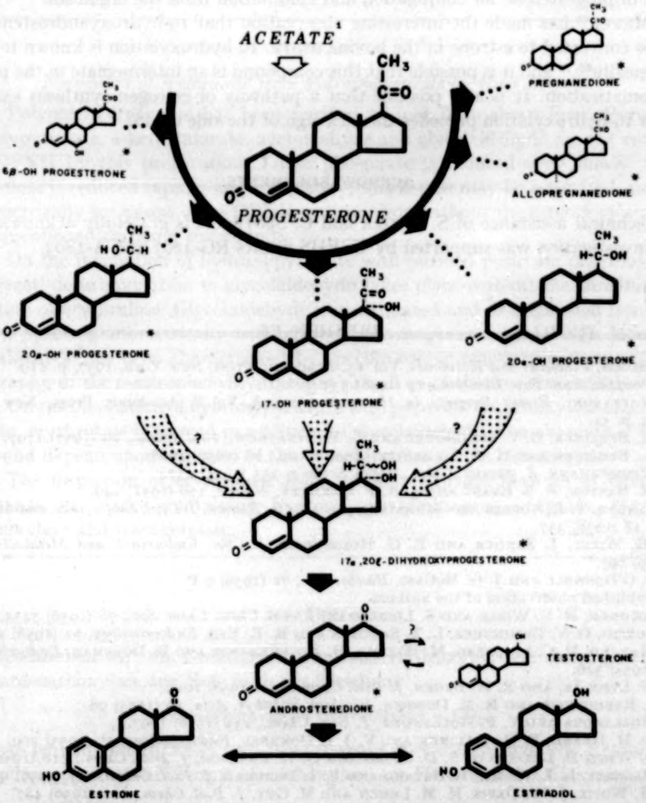
ACETATE 1
CH3
C=O
PROGESTERONE
PREGNANEDIONE *
ALLOPREGNANEDIONE *
6β-OH PROGESTERONE *
HO-C-H
20β-OH PROGESTERONE *
H-C-OH
20α-OH PROGESTERONE *
OH
17-OH PROGESTERONE *
?
?
H-C-OH
OH
17α,20ξ-DIHYDROXYPROGESTERONE
?
OH
TESTOSTERONE
ANDROSTENEDIONE *
HO
ESTRONE
HO
ESTRADIOL

Fig. 4. The metabolism of progesterone in the human ovary (proposed sequence of biosynthesis).
[1] The conversion of acetate to progesterone was demonstrated only in bovine corpora lutea.
* Compounds isolated after incubation of [4-^{14}C]progesterone in human ovarian tissue. The estrogens were isolated after incubation of androstenedione.

in Fig. 4) is not known. These compounds may be associated with the mechanism of other species. Solomon *et al.*[12] have identified androstenedione, and 17-hydroxyprogesterone after the *in vitro* incubation of progesterone in bovine ovarian tissue. Two laboratories[13,14] have reported 20β-hydroxypregnene-3-one to be a product of progesterone metabolism in the bovine ovary. A similar pattern of progesterone metabolism to that observed in the ovary appears to occur in the rat and guinea pig testis[15]. The overall synthesis of estrogens from $[^{14}C]$acetate incubated with ovarian tissue has been reported by Rabinowitz and Dowben[16]. However, Hollander and Hollander[17] were unsuccessful in demonstrating these conversions in similar action of progesterone or may be decomposition products in the course of the breakdown of progesterone for conjugation and elimination from the organism.

Meyer[24] has made the interesting observation that 19-hydroxyandrostenedione can be converted to estrone in the bovine ovary. 19-hydroxylation is known to occur biologically[25,26] and it is possible that this compound is an intermediate in the process of aromatization. It is also possible that a pathway of estrogen synthesis exists in which 19-hydroxylation precedes the cleavage of the side chain.

ACKNOWLEDGEMENTS

The technical assistance of S. Jutila and C. Slotboom is gratefully acknowledged. This investigation was supported by USPHS grants RG-4207 and A-1803.

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BILDUNG VON ERYTHRULOSE AUS HYDROXYPYRUVAT DURCH HEFEENZYMEN

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(Eingegangen am 3. August, 1959)

SUMMARY

Formation of erythrulose from hydroxypyruvate by yeast enzymes

1. Polyoldehydrogenase has been purified 96-fold from sheep liver. Pyruvate, hydroxypyruvate, α-ketoglutarate, acetaldehyde and glycolaldehyde are not reduced with DPNH by this preparation. Triose phosphate is reduced very slowly. Since erythrulose is reduced rapidly and completely, this ketose may be estimated spectrophotometrically by means of its DPNH consumption without the interference of the above mentioned carbonyl compounds.

2. On the incubation of hydroxypyruvate with purified pyruvate decarboxylase from yeast, decarboxylation to glycolaldehyde takes place without the simultaneous formation of erythrulose. Glycolaldehyde was estimated and distinguished from erythrulose spectrophotometrically with alcohol dehydrogenase and DPNH. Moreover glycolaldehyde has been characterized by specific colour reactions and paper chromatography of the *o*-nitrophenylhydrazones as well as of the free compounds.

3. On the incubation of hydroxypyruvate with pyruvate decarboxylase and transketolase, erythrulose is formed in addition to glycolaldehyde. The amounts of either compound depend upon the ratio of the concentrations of the two enzymes.

4. The formation of erythrulose from hydroxypyruvate may be of biological importance in tissues containing hydroxypyruvate-forming enzymes, pyruvate decarboxylase and transketolase.

EINFÜHRUNG

1955 beschrieben wir[1] die Decarboxylierung von Hydroxypyruvat durch gereinigte Pyruvatdecarboxylase aus Hefe nach der Gleichung

$$\text{Hydroxypyruvat} \rightarrow \text{Glycolaldehyd} + CO_2 \qquad (1)$$

1956 berichteten DICKENS UND WILLIAMSON[2] über die Bildung von Erythrulose aus Hydroxypyruvat mit Pyruvatdecarboxylase aus Hefe nach der Gleichung

$$2\ \text{Hydroxypyruvat} \rightarrow \text{Erythrulose} + 2CO_2 \qquad (2)$$

Es werden folgende Abkürzungen verwendet: DPN, Diphosphopyridinnucleotid; DPNH, hydriertes Diphosphopyridinnucleotid; HOBTS, Hydroxypyruvat; ADH, Alkoholdehydrogenase; MDH, Milchsäuredehydrogenase; Trispuffer, Trishydroxymethylaminomethanpuffer.

Freier Glycolaldehyd als möglicherweise auftretendes Zwischenprodukt der Reaktion konnte von DICKENS UND WILLIAMSON[2] nicht nachgewiesen werden.

Die vorliegende Arbeit wurde unternommen, um das Zustandekommen dieser gegensätzlichen Befunde zu klären. Wir gingen bei unseren Versuchen von der Arbeitshypothese aus, dass die unter (2) angegebene Reaktion durch Zusammenwirken der Enzyme Pyruvatdecarboxylase und Transketolase nach folgenden Gleichungen verläuft:

$$\text{Hydroxypyruvat} \xrightarrow{\text{(Pyruvatdecarboxylase)}} \text{Glycolaldehyd} + CO_2 \tag{3}$$

$$\text{Hydroxypyruvat} + \text{Glycolaldehyd} \xrightarrow{\text{(Transketolase)}} \text{Erythrulose} + CO_2 \tag{4}$$

$$\text{Summe:} \quad 2\ \text{Hydroxypyruvat} \longrightarrow \text{Erythrulose} + 2\ CO_2 \tag{5}$$

Durch die im folgenden dargestellten Versuche mit gereinigter Pyruvatdecarboxylase und kristallisierter Transketolase aus Hefe wurde diese Arbeitshypothese bestätigt.

Nach Abschluss unserer Experimente sahen wir eine Arbeit[3], in der DICKENS Daten über die Verunreinigung seiner Carboxylase-Präparationen mit Transketolase mitteilt. Aufgrund dieser Daten zieht nun auch DICKENS eine Beteiligung von Transketolase an der Erythrulose-Bildung aus Hydroxypyruvat mit Hefe-Pyruvatdecarboxylase in Betracht.

METHODEN UND PRÄPARATE

Darstellung von L-Erythrulose

Erythrulose wurde durch aerobe Vergärung von meso-Erythrit mit Sorbose-Bakterien nach BERTRAND[4] gewonnen*. Nach dem Verfahren von MÜLLER und Mitarbeiter[5] wurde Erythrulose aus dem Gäransatz als *o*-Nitrophenylhydrazon gefällt. Nach Umkristallisieren aus absolutem Äthanol betrug der Schmelzpunkt 151°C und die spezifische Drehung in absolutem Äthanol bei 20°C + 47.4° (Konzentration 10 mg/ml) in Übereinstimmung mit früheren Angaben anderer Autoren[2,5]. Freie Erythrulose wurde durch Zerlegung des *o*-Nitrophenylhydrazons mit Benzaldehyd nach den Angaben von MÜLLER und Mitarbeiter[5] als Sirup erhalten. Die spezifische Drehung betrug + 11.1° (Messungen mit 23 bzw. 101 mg/ml; Temperatur 20° C); nach MÜLLER und Mitarbeiter beträgt die Drehung + 11.4°.

Enzymatisch-optische Bestimmung von Erythrulose mit Polyoldehydrogenase

Vorkommen von Polyoldehydrogenase: In Tabelle I ist die Aktivität von Extrakten aus Leber verschiedener Tiere und von Bullen-Samenblasen bei der Reduktion von Fructose mit DPNH wiedergegeben. Man sieht aus der Tabelle, dass ein Extrakt aus Hammelleber die höchste Aktivität pro Milliliter und die höchste spezifische Aktivität des Enzyms enthält. Wir haben deshalb Hammelleber als Ausgangsmaterial für die Präparation des Enzyms benutzt. Dem Vorschlag einiger früherer Bearbeiter[6-9] des Enzyms folgend, bezeichnen wir in der vorliegenden Arbeit das Enzym als "Polyoldehydrogenase".

Reinigung und Charakterisierung der Polyoldehydrogenase aus Hammelleber: Da die bisher beschriebenen angereicherten Polyoldehydrogenase-Präparate nicht ge-

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* Der Firma Hoffmann-La Roche, Grenzach/Baden, insbesondere Herrn Dr. COUTELLE, danken wir für die Durchführung des Gäransatzes.

TABELLE I

POLYOLDEHYDROGENASE IN ORGANEN VERSCHIEDENER TIERE

Es wurden Extrakte aus Acetontrockenpulver getestet (Herstellung siehe METHODEN UND PRÄPARATE). Definition der Aktivitätseinheiten siehe METHODEN UND PRÄPARATE. Ansätze zum optischen Test: 0.066 *M* Triäthanolaminpuffer, pH, 7.4; 0.5 mg DPNH; 0.016 *M* Fructose; 0.04 ml Extrakt; *d*, 1 cm, Gesamtvol., 3 ml, Wellenlänge, 366 mμ.

	Aktivitätseinheiten/ml Rohextrakt	*mg Protein/ml Rohextrakt*	*Spezifische Aktivität*
Ratte (Leber)	360	21.0	17.1
Rind (Leber)	415	17.0	24.4
Pferd (Leber)	575	22.1	26.0
Schwein (Leber)	590	21.0	28.0
Hammel (Leber)	970	17.3	49.0
Taube (Leber)	75	20.2	3.68
Bulle (Samenblasen)	200	19.0	10.5

nügend rein und aktiv waren, um eine einigermassen spezifisch und nicht zu träg ablaufende Bestimmung von Erythrulose durchzuführen, haben wir das Anreicherungsverfahren von WILLIAMS-ASHMAN UND BANKS[10] weiter ausgebaut. Wir erhielten (allerdings mit geringer Ausbeute) eine 96fache Anreicherung des Enzyms (vgl. Tabelle II). Das derart gereinigte Enzym ist für die im folgenden beschriebene enzymatisch-optische Bestimmung von L-Erythrulose geeignet.

Im einzelnen erfolgte die Aufarbeitung der Hammellebern folgendermassen (vgl. Tabelle II): Frische Hammellebern wurden mit dem 20fachen Volumen an kaltem Aceton (—15°) im Starmix zerkleinert, auf der Nutsche abgesaugt, noch einmal mit Aceton im Starmix durchgerührt und nach Absaugen 60 min auf Filtrierpapier an der Luft (Zimmertemperatur) getrocknet. Das Acetontrockenpulver hält sich *ca.* 4 Wochen ohne nennenswerten Aktivitätsverlust im Kühlraum.

50 g Acetontrockenpulver wurden mit 500 ml destilliertem Wasser extrahiert,

TABELLE II

ANREICHERUNG DER POLYOLDEHYDROGENASE AUS HAMMELLEBER

(Definition der Aktivitätseinheiten siehe METHODEN UND PRÄPARATE) Ansätze zum optischen Test: 0.066 *M* Triäthanolaminpuffer, pH, 7.4; 0.5 mg DPNH; 0.16 *M* Fructose; *d*, 1 cm, Gesamtvolumen, 3 ml, Wellenlänge, 366 mμ.

Reinigungsstufe	*Spezifische Aktivität (Einheiten/mg Protein)*	*Ausbeute bezogen auf Rohextrakt in %*
Rohextrakt aus Acetontrockenpulver	$2.9 \cdot 10^2$	100
Säurefällung*	$2.3 \cdot 10^2$	67
Äthanolfällung	$7.2 \cdot 10^2$	42
Acetonfällung	$8.2 \cdot 10^2$	30.5
Al–Cγ–Gel	$23.6 \cdot 10^2$	27.6
DEAE-SF-Cellulose	$78 \cdot 10^2$	23.4
Äthanolfällung	$147 \cdot 10^2$	8.6
Ammonsulfatfällung	$278 \cdot 10^2$	7.9

* Bei diesem Schritt werden gefärbte Proteide und der Hauptteil der Alkoholdehydrogenase entfernt.

indem 10 min im Mörser bei Zimmertemperatur zerrieben und dann weitere 30 min mechanisch gerührt wurde. Wir zentrifugierten 30 min bei 27,000 × g und 0°. Im klaren Überstand wurde mit 1 *N* Essigsäure pH 4.65 eingestellt, hierauf 20 min bei 27,000 × g (Temperatur, 0°) zentrifugiert und im Überstand mit 1 *N* NH_4OH pH 7.8 eingestellt. Nun wurde wieder 20 min bei 27,000 × g zentrifugiert (Temperatur = 0°) und dem Überstand bei —15° langsam absolutes Äthanol bis zu einem Gehalt von 45 % (v/v) zugefügt. Dann wurde 20 min bei 27,000 × g zentrifugiert (Temperatur, 0°) und der Äthanolgehalt des Überstandes langsam bei —15° auf 55 % (v/v) erhöht. Nach dem Zentrifugieren (20 min bei 27,000 × g; Temperatur = 0°) wurde der Überstand verworfen und das Sediment in soviel 0.01 *M* Phosphatpuffer pH 7.4, gelöst, dass eine Proteinkonzentration von 10 bis 15 mg/ml vorlag. Bei —15° wurde nun dasselbe Volumen Aceton zugegeben. Nach Abzentrifugieren bei 27,000 × g (Temperatur = 0°) liess sich das Sediment im Exsikkator bei —20° ohne wesentlichen Verlust an Aktivität über Nacht aufbewahren.

Am nächsten Tag wurde das Sediment in 45 ml 0.01 *M* Phosphatpuffer, pH 7.4, aufgenommen und klar zentrifugiert. Der Überstand enthielt etwa 20 mg Protein pro Milliliter. Dazu wurde das doppelte Volumen eines *ca.* 6 Monate alten Al-Cγ-Gels[11] mit 20 mg Trockengewicht pro Milliliter gegeben. Nach 15 min Rühren wurde 30 min bei 27,000 × g (Temperatur, 0°) zentrifugiert. Der Überstand (*ca.* 120 ml) wurde auf eine mit 20 g DEAE-SF-Cellulosepulver der Firma Serva (Nr. 1709 58; 0.4 mÄquivalente/g) beschickte Säule (7 cm × 2 cm) gegeben, welche vorher mit 0.01 *M* Phosphatpuffer, pH 7.4, behandelt worden war. Dann wurde weiter 0.01 *M* Phosphatpuffer, pH 7.4, auf die Säule gegeben und 15-ml Fraktionen aufgefangen. Die die Aktivität enthaltenden Fraktionen (meist die 3. bis 10. Fraktion) wurden vereinigt. Die Enzymlösung wurde bei —16° mit Äthanol bis zu einer Endkonzentration von 55 % (v/v) versetzt. Nach dem Zentrifugieren (45 min bei 27,000 × g, Temperatur, 0°) wurde der Überstand verworfen und das Sediment in wenig 0.01 *M* Trishydroxymethylaminomethanpuffer, pH 7.4, aufgenommen und durch Zugabe von fein pulverisiertem Ammonsulfat zu 40 % gesättigt. Eine geringe Trübung wurde abzentrifugiert. Nun wurde die Ammonsulfatsättigung im Überstand auf 60 % erhöht. Dabei trat starker Seidenglanz auf. Nun wurde bei 27,000 × g 45 min zentrifugiert (Temperatur, 0°) und das Sediment in wenig 0.01 *M* Trispuffer, pH 7.4, aufgenommen. Dieses Präparat ist bei —20° mehrere Monate ohne Aktivitätsverlust haltbar.

Derart gereinigte Polyoldehydrogenase reduzierte Fructose und Erythrulose (Endkonzentration 0.01 *M*) nur mit DPNH, nicht mit TPNH. Weder nach Cysteinzusatz (Endkonzentration 0.001 *M*) noch nach Zusatz von Magnesiumchlorid (Endkonzentration 0.005 *M*) beobachteten wir eine Erhöhung der Reaktionsgeschwindigkeit (vgl. hierzu HICKMAN UND ASHWELL[12]). Unser Präparat reduzierte mit DPNH und 0.01 *M* Substratkonzentration folgende Verbindungen nicht: Pyruvat, Hydroxypyruvat, α-Ketoglutarat (auch bei Gegenwart von NH_4^+-Ionen), Acetaldehyd und Glycolaldehyd. Ein Gemisch von Phosphoglycerinaldehyd und Dihydroxyacetonphosphat wurde mit etwa 3 % der Geschwindigkeit von 0.01 *M* Erythrulose reduziert. Das Enzym eignet sich demnach zur enzymatisch-optischen Bestimmung von Erythrulose ohne Störung durch Glycolaldehyd, Hydroxypyruvat, Pyruvat, α-Ketoglutarat und Acetaldehyd.

Bestimmung von Erythrulose neben Glycolaldehyd und Hydroxypyruvat: Die

Abhängigkeit der Reaktionsgeschwindigkeit von Polyoldehydrogenase mit L-Erythrulose einerseits und Fructose andererseits als Funktion der Substratkonzentration ist nach dem Verfahren von LINEWEAVER UND BURK[13] in Fig. 1 wiedergegeben. Als Michaeliskonstanten ergaben sich für Erythrulose $2.5 \cdot 10^{-2}$ Mole/l, für Fructose 25 bis $30 \cdot 10^{-2}$ Mole/l. Die Affinität des Enzyms zu Erythrulose ist demnach eine Zehnerpotenz grösser als zu Fructose, jedoch immer noch recht gering. Für eine quantitative Erfassung von Erythrulose müssen deshalb hohe Konzentrationen des Enzyms eingesetzt werden, um in kurzer Zeit einen vollständigen Reaktionsablauf zu erhalten. Die Reinheit des Enzyms muss gross sein, damit keine Nebenreaktionen erfolgen. Mit dem im vorherigen Abschnitt beschriebenen Enzympräparat sind diese Voraussetzungen gegeben. In Fig. 2 ist ein Beispiel für die Bestimmung von Erythrulose neben Hydroxypyruvat und Glycolaldehyd wiedergegeben. Zwar reagiert unser Polyoldehydrogenase-Präparat nicht mit Hydroxypyruvat und Glycolaldehyd, sicherheitshalber wurden aber diese beiden Substanzen bei unseren Routinetesten vor der Erythrulose bestimmt: Hydroxypyruvat mit kristallisierter Milchsäuredehydrogenase aus Kaninchenmuskel und Glycolaldehyd mit kristallisierter Alkoholdehydrogenase aus Hefe wie früher angegeben[1].

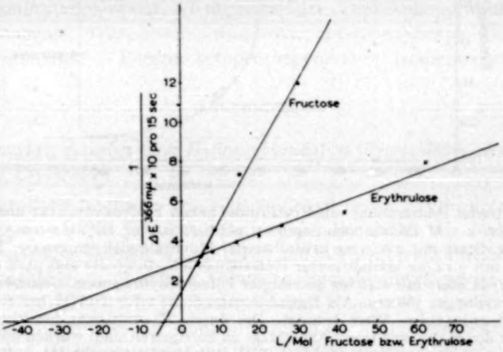

Fig. 1. Michaeliskonstanten für Fructose und Erythrulose bei der Umsetzung an Polyoldehydrogenase mit DPNH. Auswertung nach LINEWEAVER AND BURK[13]. Zusammensetzung des Testsystems wie in Tabelle II beschrieben.

Inkubation von Hydroxypyruvat mit verschiedenen Enzympräparationen

Alle Ansätze wurden bei 28–30° in Citratpuffer, pH 6.8, (Endkonzentration 0.1 *M*) bzw. in Trishydroxymethylaminomethanpuffer, pH 7.4, (Endkonzentration 0.05 *M*) durchgeführt, Inkubationszeit 5 Stunden. Die Proben wurden 5 min bei 80° erhitzt, nach dem Abkühlen 3 min bei 2000 × *g* zentrifugiert und der Überstand mit dem enzymatisch-optischen Test bzw. durch Papierchromatographie analysiert.

Angaben über die verwendeten Enzyme und Präparate

Pyruvatdecarboxylase wurde aus getrockneter Bierhefe der Ganter-Brauerei (Freiburg im Breisgau) nach einem früher angegebenen Verfahren[15] gereinigt. Kristallisierte Alkoholdehydrogenase wurde aus getrockneter Bäckerhefe der Sinner AG. (Karlsruhe) nach RACKER[16] präpariert. Kristallisierte Transketolase präparierte

Herr Dr. Busch in unserem Institut nach den Angaben von Srere und Mitarbeiter[17] aus getrockneter Bäckerhefe. Kristallisierte Milchsäuredehydrogenase aus Kaninchenmuskel wurde von der Firma Boehringer (Mannheim) bezogen, ebenso Triosephosphatisomerase und α-Glycerophosphatdehydrogenase. Weiter lieferte Boehringer 85 %iges DPN und ca 78 %iges enzymatisch reduziertes DPNH. Phosphoriboseisomerase und Phosphoketopentoepimerase ("Isomerasegemisch") wurden aus Bäckerhefe nach Tabachnik und Mitarbeiter[18] hergestellt. Macerationssaft wurde nach der Vorschrift von Lebedew[19] aus getrockneter Bierhefe der Ganter-Brauerei (Freiburg) bereitet; Macerationssaft aus getrockneter Bäckerhefe der Sinner AG. (Karlsruhe) gewannen wir nach der Vorschrift von Racker[16].

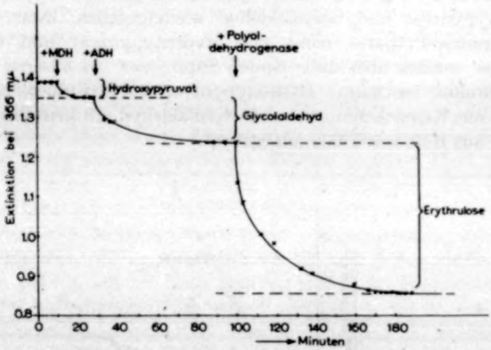

Fig. 2. Enzymatische Bestimmung von Erythrulose neben Hydroxypyruvat und Glycolaldehyd. Ansatz zum Test: 0.1 *M* Triäthanolaminpuffer, pH 7.4; 0.3 mg DPNH; 0.015 ml der zu analysierenden Probe. Start mit 0.025 mg kristallisierter Milchsäuredehydrogenase. Nach Ablauf der Reaktion Start mit 0.12 mg kristallisierter Hefealkoholdehydrogenase und nach der Bestimmung des Glycolaldehyds Start mit 0.18 mg gereinigter Polyoldehydrogenase. Gesamtvolumen, 0.4 ml; *d*, 0.5 cm; Wellenlänge, 366 mμ. Als Extinktionskoeffizient für DPNH bei 366 mμ wurde der von Hohorst[14] angegebene Wert benutzt. Die durch Enzymzusatz bewirkten Extinktionsdifferenzen (mit denen die erhaltenen Ausschläge zu korrigieren sind) wurden durch nochmaligen Zusatz des Enzyms nach Reaktionsablauf ermittelt.

Hydroxypyruvat wurde nach Sprinson und Chargaff[20] hergestellt. Glycolaldehyd bezogen wir von den Firmen Light (England) und Fluka AG. (Buchs, SG Schweiz). Ebenso wurde Ribose-5-phosphat als Bariumsalz von der Fluka AG. geliefert. *o*-Nitrophenylhydrazin wurde aus *o*-Nitroanilin durch Diazotierung nach Müller und Mitarbeiter[5] und Bischler[21] dargestellt. Thiaminpyrophosphat und 2,4-Dinitrophenylhydrazin bezogen wir von der Firma Merck (Darmstadt).

Darstellung der o-Nitrophenylhydrazone als Vergleichssubstanzen für die Papierchromatographie

Hydroxypyruvat, Glycolaldehyd bzw. Erythrulose wurden jeweils in wenig Methanol gelöst und in heissem Methanol gelöstes *o*-Nitrophenylhydrazin in geringem Überschuss zugefügt. Nach 10 min Erwärmen bei 80° fiel beim anschliessenden Abkühlen das *o*-Nitrophenylhydrazon aus. Es wurde abgesaugt, mit Methanol, dann mit Äther gewaschen, auf dem Tonteller abgepresst und aus absolutem Äthanol umkristallisiert. Der Schmelzpunkt des *o*-Nitrophenylhydrazons von Glycolaldehyd betrug 116–117°, der des *o*-Nitrophenylhydrazons von Erythrulose 151°. Zur Gewinnung der *o*-Nitrophenylhydrazone aus den enzymatischen Ansätzen wurden diese durch Erwärmen auf 80° enteiweisst, mit einem Überschuss von *o*-Nitrophenylhydrazin versetzt und 40 min bei 60° erwärmt. Nach dem Abkühlen wurde abgesaugt und wie oben beschrieben, gewaschen und umkristallisiert. Zur Chromatographie nach MEIGH[22] wurden die *o*-Nitrophenylhydrazone in wenig 96 %igem Äthanol gelöst und auf das Papier aufgetragen.

Definition der in dieser Arbeit benutzten Enzymaktivitätseinheiten

Als 1 Einheit wird eine Änderung der Extinktion um 0.001/min bei 366 mμ im optischen Test mit DPNH bei einem Gesamtvolumen des Testansatzes von 3.0 ml und einer Schichtdicke von 1 cm bezeichnet. Die Temperatur war 18–22°. Beim Test der Pyruvatdecarboxylase wurde mit 0.12 *M* Citratpuffer, pH 6.0, gearbeitet, die Pyruvatkonzentration war 0.025 *M*. Transketolase wurde im Prinzip nach DE LA HABA und Mitarbeitern[23] in 0.05 *M* Trishydroxymethylaminomethanpuffer, pH 7.6, getestet. Der Ansatz enthielt 8.2 μMole Ribose-5-phosphat, 9 μMole Magnesiumchlorid, 0.12 μMol Thiaminpyrophosphat, 0.6 mg reduziertes Diphosphopyridinnucleotid und folgende Hilfsenzyme: Triosephosphatisomerase, α-Glycerophosphatdehydrogenase, Phosphoriboseisomerase + Phosphoketopentoepimerase ("Isomerasegemisch").

ERGEBNISSE

Pyruvatdecarboxylase decarboxyliert Hydroxypyruvat zu Glycolaldehyd ohne gleichzeitige Entstehung von Erythrulose

Zum qualitativen Nachweis von Glycolaldehyd neben Erythrulose in Ansätzen mit Hydroxypyruvat und Pyruvatdecarboxylase benutzten wir fuchsinschweflige Säure (0.1 % Fuchsin in heissem Wasser gelöst und mit SO_2 bis zur Entfärbung begast). Durch Zusammenbringen desselben Volumens Fuchsinschwefligsäure-Reagenz und Probe konnte Glycolaldehyd in Konzentrationen bis herunter zu $2 \cdot 10^{-3}$ Mol/l nachgewiesen werden. Erythrulose in Konzentrationen bis 1 Mol/l ergab keine Farbreaktion. Ansätze von Hydroxypyruvat mit Pyruvatdecarboxylase ergaben nach Enteiweissen durch Erhitzen und Versetzen mit dem Reagenz eine starke Farbreaktion. Auswertung der Farbintensität von Verdünnungsreihen ergab Glycolaldehydkonzentrationen der Grössenordnung, wie sie aufgrund der enzymatisch-optischen Bestimmung von Glycolaldehyd mit Hefealkoholdehydrogenase (vgl. Tabelle IV) zu erwarten waren.

Nach DICKENS UND WILLIAMSON[24] ergibt Glycolaldehyd mit Naphtoresorcin in stark schwefelsaurem Milieu bereits in der Kälte eine grün-gelbe Färbung. Erythrulose reagiert unseren Messungen zufolge unter diesen Bedingungen nicht. Ansätze von Hydroxypyruvat mit reiner Pyruvatdecarboxylase ergaben eine positive Naphthoresorcinreaktion. Wie beim Test mit fuchsinschwefliger Säure ergab eine halbquantitative Auswertung der Farbintensität Glycolaldehydkonzentrationen in der Grössenordnung der im optischen Test mit Alkoholdehydrogenase erhaltenen Konzentrationen.

Zur weiteren Identifizierung des Reaktionsproduktes beim Umsatz von Hydroxypyruvat mit Pyruvatdecarboxylase wurden die durch Erhitzen auf 80° enteiweissten

Ansätze entweder direkt oder nach Überführung der darin enthaltenen Carbonylverbindungen in *o*-Nitrophenylhydrazone bzw. 2,4-Dinitrophenylhydrazone nach MEIGH[22] papierchromatographiert. In Tabelle III sind die R_F-Werte der bei der Chromatographie erhaltenen Flecke wiedergegeben. Man sieht aus Tabelle III, dass nach Umsatz von Hydroxypyruvat mit Pyruvatdecarboxylase allein Glycolaldehyd bzw. sein *o*-Nitrophenylhydrazon nachweisbar ist, während in Ansätzen mit rohem Macerationssaft aus Bierhefe als Enzymquelle vorwiegend Erythrulose bzw. ihr *o*-Nitrophenylhydrazon gefunden wird. Auf die Ergebnisse mit Macerationssaft werden wir im nächsten Abschnitt zurückkommen; hier sei festgehalten, dass mit reiner Pyruvatdecarboxylase im Einklang mit den vorstehend mitgeteilten Farbreaktionen allein Glycolaldehyd als Reaktionsprodukt aufgefunden wurde. Unsere früheren papierchromatographischen Versuche mit den 2,4-Dinitrophenylhydrazonen[1] waren für eine Unterscheidung von Glycolaldehyd und Erythrulose nicht geeignet, da die Dinitrophenylhydrazone dieser Verbindungen nicht getrennt werden (vgl. Tabelle III).

TABELLE III

R_F-WERTE VON GLYCOLALDEHYD, ERYTHRULOSE UND HYDROXYPYRUVAT, SOWIE IHRER *o*-NITROPHENYLHYDRAZONE UND 2,4-DINITROPHENYLHYDRAZONE

Absteigende Chromatographie; mobile Phase: mit Heptan gesättigtes Methanol[22]; Papier: Schleicher und Schüll 2043 b Mgl. Die Phenylhydrazone wurden durch ihre gelbe Farbe erkannt, die freien Carbonylverbindungen wurden durch Besprühen mit einer gesättigten Lösung von *m*-Phenylendiamin in absolutem Äthanol und 10 min Erhitzen auf 110° sichtbar gemacht.

	R_F-Werte der unbehandelten Substanzen bzw. Inkubationsansätze	*R_F-Werte nach Überführung in o-Nitrophenylhydrazone*	*R_F-Werte nach Überführung in 2,4-Dinitrophenylhydrazone*
Glycolaldehyd (authentische Probe)	0.83	0.77	0.66
Erythrulose (authentische Probe)	0.72	0.69	0.66
Hydroxypyruvat (authentische Probe)	0.57	0.62	0.46
Hydroxypyruvat mit Pyruvatdecarboxylase inkubiert	0.83	0.77	—
Hydroxypyruvat mit Macerationssaft aus Bierhefe inkubiert	0.72	0.69	—

In unserer früheren Arbeit wiesen wir Glycolaldehyd durch quantitative Reduktion mit DPNH an kristallisierter Alkoholdehydrogenase aus Hefe nach. Wir prüften nun, ob auch Erythrulose unter diesen Bedingungen reduziert wird. In einem Ansatz mit 0.22 *M* Citratpuffer, pH 6.0, 0.6 mg DPNH und einer Endkonzentration von $0.56 \cdot 10^{-2}$ *M* Erythrulose nach Zusatz von 0.3 mg kristallisierter Hefe-Alkoholdehydrogenase wurde kein Verschwinden von DPNH beobachtet (Gesamtvolumen 3.0 ml Schichtdicke 1 cm; Wellenlänge 366 mμ). Wurden dem Ansatz 0.5 μMol Glykolaldehyd zugesetzt, so fand rasche und quantitative Hydrierung des Glycolaldehyds unter Verbrauch von DPNH statt. Der in unserer früheren Arbeit beschriebene Verbrauch von DPNH nach Zusatz von Hefealkoholdehydrogenase zu Ansätzen mit Hydroxypyruvat und Pyruvatdecarboxylase kann demnach nicht auf die Bildung von Erythrulose zurückgeführt werden.

In Fig. 3 ist ein Versuch wiedergegeben, der zeigt, dass die beim Umsatz von Hydroxypyruvat mit Pyruvatdecarboxylase entstehende Substanz quantitativ mit DPNH und Alkoholdehydrogenase aus Hefe reduziert wird. Kurve 1 wurde mit einer

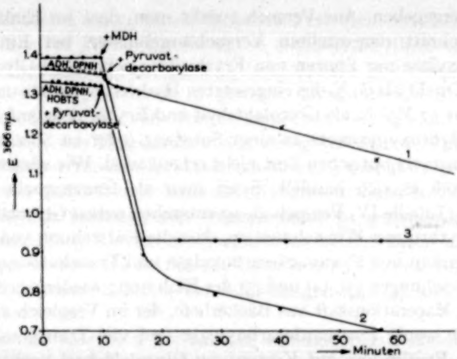

Fig. 3. Quantitativer Umsatz von Hydroxypyruvat mit Pyruvatdecarboxylase zu einer Verbindung, die mit Alkoholdehydrogenase und DPNH reduziert wird. Ansätze zum optischen Test: 0.22 *M* Citratpuffer, pH 6.0 (Versuche 1 und 2), bzw. 0.133 *M* Trishydroxymethylaminomethanpuffer, pH 8.0 (Versuch 3); 1 mg DPNH; 0.43 μMol Hydroxypyruvat; 0.6 mg kristallisierte Alkoholdehydrogenase bzw. 0.05 mg kristallisierte Milchsäuredehydrogenase. Gesamtvolumen: 3.0 ml; *d*, 1 cm; Wellenlänge, 366 mμ.

Kontrollküvette erhalten, in der Alkoholdehydrogenase, DPNH und Pyruvatdecarboxylase, jedoch kein Hydroxypyruvat enthalten war. Kurve 2 gibt denselben Versuch mit Zusatz von Hydroxypyruvat wieder. Als Differenz aus den Kurven 1 und 2 errechnet man einen Verbrauch von 0.416 μMol DPNH, der durch den Umsatz von Hydroxypyruvat mit Pyruvatdecarboxylase bewirkt wurde. Kurve 3 gibt die Austestung des eingesetzten Hydroxypyruvats mit DPNH und kristallisierter Milchsäuredehydrogenase aus Kaninchenmuskel wieder. Aus der DPNH-Abnahme berechnet man 0.42 μMol Hydroxypyruvat im Ansatz. Da der DPNH-Verbrauch im Versuch mit Pyruvatdecarboxylase ebenso gross war wie der DPNH-Verbrauch durch Reduktion von Hydroxypyruvat mit Milchsäuredehydrogenase, beweist dieser Versuch, dass beim Umsatz von Hydroxypyruvat mit Pyruvatdecarboxylase in stöchiometrischer Menge zum Hydroxypyruvat eine Substanz entsteht, die mit DPNH an Alkoholdehydrogenase reduziert wird. Da Erythrulose nicht mit DPNH an Alkoholdehydrogenase reduziert wird, kann es sich nicht um diese Tetrose handeln. Den vorstehend mitgeteilten Versuchen zufolge kommt als Reaktionsprodukt nur Glycolaldehyd in Frage, sodass die früher von uns formulierte Gleichung (1) bestätigt ist.

Entstehung von Erythrulose aus Hydroxypyruvat

Im Kapitel METHODEN beschreiben wir einen enzymatisch-optischen Test für Erythrulose mit Polyoldehydrogenase aus Hammelleber. Das gereinigte Enzym reagiert nicht mit Hydroxypyruvat und Glycolaldehyd. Da Hydroxypyruvat mit DPNH und Milchsäuredehydrogenase aus Kaninchenmuskel und Glycolaldehyd mit Hefe-Alkoholdehydrogenase bestimmt werden kann, ist eine schnelle und spezifische Nebeneinanderbestimmung dieser 3 Substanzen im enzymatisch-optischen Test möglich (vgl. Fig. 2). Wir haben mit dieser Methode Ansätze von Hydroxypyruvat mit verschiedenen Enzympräparationen analysiert. Die Ergebnisse sind in

Tabelle IV wiedergegeben. Aus Versuch 1 sieht man, dass im Einklang mit den im vorherigen Abschnitt dargestellten Versuchsergebnissen bei Einsatz von reiner Pyruvatdecarboxylase nur Spuren von Erythrulose neben viel Glycolaldehyd nachweisbar sind. Da mehr als 99 % des eingesetzten Hydroxypyruvats umgesetzt wurden, davon jedoch nur 57 Mol % als Glycolaldehyd und Erythrulose aufgefunden wurden, sind 43 % des Hydroxypyruvats zu einer Substanz (oder zu Substanzen) verändert worden, die in unserem optischen Test nicht erfasst wird. Wir wissen noch nicht, um welche Substanzen es sich handelt. Setzt man als Enzymquelle Macerationssaft aus Bierhefe ein (Tabelle IV, Versuch 2), so entstehen neben Glycolaldehyd beträchtliche Mengen Erythrulose. Wir nehmen an, dass die Entstehung von Erythrulose auf das Zusammenwirken von Pyruvatdecarboxylase und Transketolase zurückzuführen ist, wie in den Gleichungen (3), (4) und (5) der Einleitung wiedergegeben wurde. Dafür spricht, dass ein Macerationssaft aus Bäckerhefe, der im Vergleich zum Macerationssaft aus Bierhefe wenig Pyruvatdecarboxylase und viel Transketolase enthält, die Entstehung von Erythrulose auf Kosten von Glycolaldehyd noch mehr begünstigt (Tabelle IV, Versuch 3). In diesem Versuch wurden nur 67 % des eingesetzten Hydroxypyruvats umgesetzt, da die geringe Pyruvatdecarboxylase-Aktivität des Bäckerhefeextraktes nur einen langsamen Hydroxypyruvat-Umsatz ermöglicht. Die den Hydroxypyruvatumsatz einleitende und in seiner Geschwindigkeit begrenzende Reaktion ist demnach die Decarboxylierung zu Glycolaldehyd. Was mit dem so entstandenen Glycolaldehyd weiter geschieht, entscheidet die anwesende Transketolase. Ist keine Transketolase vorhanden (Versuchsreihe 1), so bleibt der Hauptteil des Glycolaldehyds liegen und der Rest wird zu Produkten weiter verändert, die wir

TABELLE IV

UMSETZUNG VON HYDROXYPYRUVAT MIT VERSCHIEDENEN ENZYMPRÄPARATEN

Inkubation und Gewinnung der Proben wie unter METHODEN UND PRÄPARATE angegeben. Aktivitätseinheiten der Enzympräparate/mg Protein: gereinigte Pyruvatdecarboxylase ($2.2 \cdot 10^4$), kristallisierte Transketolase ($0.23 \cdot 10^4$), Bierhefe-Macerationssaft (Pyruvat-decarboxylase: $0.24 \cdot 10^4$), Bäckerhefe-Macerationssaft (Pyruvat-decarboxylase: $0.01 \cdot 10^4$, Transketolase: $0.004 \cdot 10^4$). Definition der Aktivitätseinheiten siehe METHODEN UND PRÄPARATE. Die Versuchsreihen 1–3 wurden in Citratpuffer, pH 6.8 (Endkonzentration 0.1 *M*), die Versuchsreihen 4–6 in Trishydroxymethylaminomethanpuffer, pH 7.4 (Endkonzentration 0.05 *M*), durchgeführt.

Versuchsreihe	*Enzympräparat*	*Mol % (bezogen auf eingesetzte HOBTS = 100 %)*			
		Glycolaldehyd	*Erythrulose**	*Glycolaldehyde + Erythrulose*	*HOBTS*
1	Gereinigte Pyruvat-decarboxylase	55	2	57	0.2
2	Macerationssaft (Bierhefe)	13	84	97	1
3	Macerationssaft (Bäckerhefe)	2	64	66	33
4	Gereinigte Pyruvat-decarboxylase und kristallisierte Transketolase	16	86	102	3
5	Kristallisierte Transketolase	4	13	17	56
6	Kristallisierte Transketolase; Glycolaldehyd zugesetzt	—	90	—	3.5

* Bei der Auswertung der Versuchsreihen 1–5 wurde je 1 Mol im Ansatz entstandener Erythrulose mit einem Verbrauch von 2 Molen Hydroxypyruvat gleichgesetzt (vgl. Gleichung (2)). Beim Versuch 6 wurde je 1 Mol im Ansatz analysierter Erythrulose mit einem Verbrauch von 1 Mol Hydroxypyruvat gleichgesetzt, da hier Glycolaldehyd im Überschuss zugesetzt worden war.

in unserem kombinierten enzymatisch-optischen Test nicht erfassen können. Liegt wenig Transketolase vor (Versuchsreihe 2), so entsteht Erythrulose neben Glycolaldehyd. Ist viel Transketolase vorhanden (Versuchsreihe 3), so entsteht vorwiegend Erythrulose; Glycolaldehyd ist nur in geringer Menge nachweisbar. Die Nebenreaktionen zu Produkten, die von unseren optischen Testen nicht erfasst werden, fallen bei Anwesenheit von Transketolase nur wenig ins Gewicht. Dies ist wohl so zu erklären, dass bei diesen Versuchen (Versuchsreihen 2, 3 und 4) Transketolase Glycolaldehyd rasch weiter umsetzt und damit den Nebenreaktionen entzieht.

Durch Zusatz von kristallisierter Transketolase zu gereinigter Pyruvatdecarboxylase (Versuchsreihe 4) können die Verhältnisse in Extrakten aus Bierhefe und Bäckerhefe imitiert werden. Da bei diesem Versuch reine Enzyme eingesetzt wurden, ist damit das Funktionieren der in den Gleichungen (3), (4) und (5) postulierten Reaktionsfolgen bewiesen.

Wie zu erwarten, entstehen bei Einsatz von reiner Transketolase nur geringe Mengen Glycolaldehyd und Erythrulose (Versuchsreihe 5). Dies ist darauf zurückzuführen, dass die Transketolase noch in geringem Masse mit Pyruvatdecarboxylase verunreinigt ist. De la Haba und Mitarbeiter[23] zeigten, dass Hydroxypyruvat an reiner Transketolase bei Gegenwart von zugesetztem Glycolaldehyd als Acceptoraldehyd umgesetzt wird. Im Einklang damit beobachtet man bei Inkubation von reiner Transketolase mit Hydroxypyruvat unter Zusatz von überschüssigem Glycolaldehyd (Versuchsreihe 6) fast vollständige Überführung von Hydroxypyruvat in Erythrulose.

DISKUSSION

Da für die in der vorliegenden Arbeit beschriebene Überführung von Hydroxypyruvat in Erythrulose neben der universell verbreiteten Transketolase Pyruvatdecarboxylase benötigt wird, kann diese Reaktion nur in pyruvatdecarboxylasehaltigen Zellen, also in Pilzen, gewissen Bakterien und Pflanzen verifiziert sein. Ob die Reaktion in diesem Material in bedeutungsvollem Umfange stattfindet, dürfte massgebend von der stationären Hydroxypyruvat-Konzentration abhängen. Virtanen und Mitarbeiter[25, 26] wiesen in pflanzlichen Geweben Hydroxypyruvat nach. Auch wir[27] konnten in früheren Untersuchungen mit einem enzymatisch-optischen Test zeigen, dass Hydroxypyruvat in Gräser-Choleoptilen in einer Konzentration von etwa 10^{-5} Mol/l vorliegt. Es ist demnach damit zu rechnen, dass in Hefen und höheren Pflanzen tatsächlich eine Erythrulose-Bildung aus Hydroxypyruvat stattfindet.

Möglichkeiten für die Synthese von Hexosen aus Erythrulose unter Beteiligung von Transketolase und Transaldolase wurden von Dickens[3] diskutiert. Nach Dickens ist demnach ein Übergang von Hydroxypyruvat in Hexosen in Betracht zu ziehen.

ZUSAMMENFASSUNG

1. Die 96fache Anreicherung von Polyoldehydrogenase aus Hammelleber wird beschrieben. Dieses Präparat reduziert mit DPNH folgende Verbindungen nicht: Pyruvat, Hydroxypyruvat, α-Ketoglutarat, Acetaldehyd und Glycolaldehyd. Triosephosphat wird nur sehr langsam reduziert. Da Erythrulose rasch und vollständig

reduziert wird, kann diese Ketose enzymatisch-optisch ohne Störung durch die andern genannten Carbonylverbindungen aufgrund des DPNH-Verbrauches bestimmt werden.

2. Inkubiert man Hydroxypyruvat mit gereinigter Pyruvatdecarboxylase aus Hefe, so findet Decarboxylierung zu Glycolaldehyd ohne gleichzeitige Bildung von Erythrulose statt. Glycolaldehyd wird in diesen Ansätzen mit Farbreaktionen, durch enzymatisch-optische Bestimmung mit Alkoholdehydrogenase und durch Papierchromatographie der *o*-Nitrophenylhydrazone, sowie der freien Verbindungen nachgewiesen und von Erythrulose unterschieden.

3. Inkubiert man Hydroxypyruvat mit Pyruvatdecarboxylase und Transketolase, so entsteht neben Glycolaldehyd Erythrulose. Das Mengenverhältnis von Glycolaldehyd zu Erythrulose hängt vom Konzentrationsverhältnis der beiden Enzyme ab.

4. Die Bildung von Erythrulose aus Hydroxypyruvat kann in Geweben, die Hydroxypyruvat bildende Enzymsysteme, Pyruvatdecarboxylase und Transketolase enthalten, biologisch bedeutsam sein.

Der Deutschen Forschungsgemeinschaft danken wir für die Unterstützung unserer Arbeiten im Rahmen des Schwerpunktprogrammes Biochemie. Der Sinner AG (Karlsruhe) und der Ganter-Brauerei (Freiburg) danken wir für die kostenlose Überlassung von Bäckerhefe und Bierhefe.

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BIOCHIMICA ET BIOPHYSICA ACTA

EFFECT OF INHIBITORS ON THE METABOLISM OF SPECIFICALLY LABELLED GLUCOSE BY BRAIN

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SUMMARY

Arsenite and menadione alter the normal metabolic pathways of guinea-pig brain cortex in a manner which may be interpreted as permitting a greater participation of the direct oxidative pathway, in contrast to the Embden-Meyerhof pathway. The means by which these two compounds accomplish this change appear to be fundamentally different. Chlorpromazine, two dodecylpyridinium compounds, iodoacetate and probably arsenate seem to be general metabolic inhibitors which have little selective influence on these two routes. Similarly, in uninhibited brain metabolism, potassium chloride stimulation of respiration or its omission have no selective influence.

INTRODUCTION

Mammalian brain is generally considered to utilize the Embden-Meyerhof glycolysis route almost exclusively[1,2]. For example, brain slices consistently give a value of approximately 1 for the ratio $^{14}CO_2$ from [6-^{14}C]G/$^{14}CO_2$ from [1-^{14}C]G. It appeared interesting, therefore, to test whether some metabolic inhibitors might be capable of dissociating this ratio by altering the metabolic pathways.

Although the use of inhibitors may give a distorted picture of normal metabolism, the interesting question underlying these studies is less one of what biochemical pathways are normally used than one of how existing pathways may be modified and, qualitatively, in view of the limitations of the present method[3], to what extent alternate pathways may be utilized.

MATERIALS AND METHODS

Metabolic experiments

Warburg vessels were used which were equipped with a center-well and two side-arms. When assembled, vessels contained the following: in one side-arm, 50 μmoles glucose (radioactive plus non-radioactive) in 0.2 ml 1.5 *M* KCl[4] (in 0.2 ml distilled water for the experiment in which KCl stimulation was omitted); in the other side-arm 0.15 ml 5 *N* sulfuric acid; in the center-well 0.15 ml 20 % KOH and a filter paper wick; and in the main compartment approx. 50 mg of guinea-pig brain cortex slices and sufficient phosphate saline buffer[4] to give a final volume of 3.0 ml. When other compounds were added, they were dissolved in the buffer and the pH was checked to maintain 7.4. At the end of a 3-h incubation period, 30°, O_2 atmosphere, the reaction was stopped by the addition of acid and the contents of the center-well were assayed as previously described[5].

Inhibitors

The following quaternary nitrogen derivatives, the abbreviations of which are given, were used: pyridine-2-aldoxime methiodide, PAM[6]; pyridine methiodide, PMI[7]; pyridine-2-aldoxime dodeciodide, PAD[8]; pyridine dodeciodide, PDI[7]. The methylated members of this series are lipid-insoluble; the dodecyl analogues, lipid-soluble. All of the pyridine derivatives were synthesized by Dr. S. GINSBURG of this Department. Chlorpromazine hydrochloride was a gift from Smith, Kline, and French Laboratories, Philadelphia, and Synkavite was kindly given by Hoffmann-La Roche Inc., Nutley, New Jersey.

The methylphosphonate diester of menadione, 2-methyl-1,4-naphthohydroquinone di(methylphosphonic acid) ester, was synthesized by the following steps. Methylphosphonyl dichloride (5 g, 0.04 mole), obtained through the courtesy of Chemical Warfare Laboratories, Army Chemical Center, Maryland, was reacted with 1.74 g (0.01 mole) of 2-methyl-1,4-dihydroxynaphthalene in an excess of anhydrous pyridine. About 15 min after mixing, a mildly exothermic reaction commenced and a white precipitate formed over a period of several hours. The reaction mixture was filtered, care being taken to avoid contact with atmospheric moisture, and the filtrate was concentrated to a pale yellow syrup at about 80° and 20 mm pressure. Ice was added to the syrup and sufficient ether to give, finally, about 1:25 ether : water. This mixture was shaken violently and, after the ice had melted, filtered. The white precipitate was washed with ether, dissolved in hot ethyl alcohol, and recrystallized on addition of ether and cooling. The product melted at 204–206° and had an equivalent weight by titration of 174.2. Another recrystallization from alcohol–ether and a recrystallization from water gave a sharp-melting white crystalline product; m.p., 204–205°, equivalent weight, 167.0. Calc. for $C_{11}H_8O_2[CH_3P(O)OH]_2$, 165.1, u.v. absorption spectrum virtually identical with that of the diphosphate ester, Synkavite.

RESULTS

The results are summarized in Table I, which shows oxygen uptake by slices of guinea-pig brain cortex, the production of $^{14}CO_2$ from [1-^{14}C]G and from [6-^{14}C]G, and the ratios $^{14}CO_2$ from [6-^{14}C]G/$^{14}CO_2$ from [1-^{14}C]G. It is known that oxygen uptake and glucose metabolism in brain slices are stimulated by an increase in the potassium ion concentration from about 0.005 *M* to about 0.1 *M*[4,9]. The difference in results between low and high potassium ion concentrations is shown in the first two lines of Table I. All of the other compounds added, shown in the next three groups of results in Table I, were tested under KCl stimulation, *i.e.*, in the higher potassium ion concentration. The first five compounds produced little or no deviation from the normal values. Chlorpromazine and iodoacetate, at the concentrations used, caused marked inhibition of both radiochemical yields and oxygen consumption. Both compounds, however, had little effect on the ratios of the radiochemical yields. Both PAD and PDI also caused marked metabolic inhibition but, again, did not greatly alter the ratio of the radiochemical yields from the two labelled substrates.

TABLE I

METABOLISM OF RADIOACTIVE GLUCOSE BY SLICES OF GUINEA-PIG BRAIN CORTEX

K ion concentration sufficient for stimulation of respiration in all experiments except the second, as indicated. First experiment average of 5 (n = 4 for calculation of standard deviation); all others single experiments. Other conditions given in text.

Compound added	*Molarity, — × 10⁻⁴*	*Radiochemical yield of $^{14}CO_2$, % per g fresh tissue, from*		*O_2 uptake, µl per g fresh tissue*	*Ratio, [6-^{14}C]G / [1-^{14}C]G*
		[1-^{14}C]G	*[6-^{14}C]G*		
None		20.3 ± 4.8	18.4 ± 5.4	3208 ± 599	0.90 ± 0.11
None, no K⁺ stim.		7.56	7.88	1948	1.04
Benzene	300	19.1	23.0	3405	1.20
PAM	5	20.2	18.5		0.92
PMI	5	19.7	15.1	3175	0.77
Dodecyl iodide*		17.1		3125	
Methylphosphonate diester of menadione	10	25.6	17.9	3005	0.70
Chlorpromazine	1	4.11	3.56	1205	0.87
Chlorpromazine	5	2.54	1.95		0.77
Iodoacetate	4	7.64	6.80	1798	0.89
PAD	5	7.81	5.37		0.69
PDI	5	6.93	5.88	1425	0.85
Arsenate	5	15.2	11.0	2780	0.72
Arsenite	2	10.9	0.47	400	0.04
Arsenite	8	2.87	0.095	828	0.03
Synkavite	5	46.4	14.8	3650	0.32
Synkavite	10	63	16.0	3840	0.25

* 1 mg per Warburg vessel; approx. $10 \cdot 10^{-4}$ *M*, had it been soluble.

Arsenate ion, at the concentration shown, appears to have a small, but similar, effect on glucose metabolism.

A very different picture was obtained with the third group of inhibitors tested. A concentration of arsenite which caused about half-inhibition of the radiochemical yield from [1-^{14}C]G caused an 88 % inhibition of oxygen uptake and virtually abolished the radiochemical yield of $^{14}CO_2$ from [6-^{14}C]G. This, in turn, reduced the ratio $^{14}CO_2$ from [6-^{14}C]G/$^{14}CO_2$ from [1-^{14}C]G from 0.90 to 0.04. A higher concentration of arsenite had a consistently similar effect except for a slightly higher oxygen uptake. Synkavite also markedly reduced the ratio of the radiochemical yields, but in a strikingly different way from that observed with arsenite. Here, there was no inhibition of oxidation or of $^{14}CO_2$ production from [6-^{14}C]G, but instead a several-fold stimulation of the radiochemical yield of $^{14}CO_2$ from [1-^{14}C]G. The oxygen uptake, although not statistically significantly higher than the average controls, was, in both cases, increased by about 20 % over the individuals controls.

DISCUSSION

It appears from these results that brain tissue, which generally metabolizes glucose essentially by the Embden-Meyerhof pathway[1,2], can be made to utilize what must be a pre-existing alternate route. Arsenite has accomplished this by the inhibition of a later common reaction, probably the one involving fumarase or the enzyme or enzymes which catalyze the oxidative decarboxylation of pyruvate to give acetyl coenzyme-A[10,11].

The means by which Synkavite has accomplished this is probably related to the uncoupling and accompanying stimulatory action[12,13] of its hydrolysis product, 2-methyl-1,4-naphthohydroquinone or the corresponding quinone, menadione. This conclusion is supported by the lack of effect of enzymically unhydrolyzable methylphosphonate diester of menadione, the latter it was found, being unhydrolyzed after 5 h incubations in liver brei or in intestinal brei, treatments which completely hydrolyzed Synkavite. Such resistance to enzyme action has been noted previously for a methylphosphonate ester[14]. It is safe to assume that the total dissociation and hydrolysis of the Synkavite, which would have increased the sodium ion content of the buffering medium by only about 3% and the phosphate content by only about 20%, respectively, would not account for the effects observed. Whether the results obtained here with Synkavite could be realized at lower concentrations or with other uncoupling agents or even with vitamin K_1 would require further investigation. However, it is noteworthy that other investigators have concluded, on the basis of results obtained with inhibitors and specifically labelled glucose, that the hexose monophosphate oxidative pathway may be involved in the intermediary metabolism of erythrocytes[15] and retinal tissue[16].

Chlorpromazine, in contrast to arsenite, appears to exercise its inhibitory action on an early common step, as has been partly indicated in studies of the effects of chlorpromazine on purified hexokinase[17].

Two predominantly lipid-soluble compounds, benzene and dodecyl iodide, and two predominantly water-soluble compounds, PAM and PMI, are without effect, so far as tested, but the lipid- and water-soluble compounds PAD and PDI are inhibitory. This effect may be accounted for by two factors: first, the greater ability of PAD and PDI to penetrate tissue[8]; and second, the ability of these compounds to react unspecifically with proteins due to Van der Waal's and Coulombic forces when applied in high concentration (unpublished results of S. EHRENPREIS AND HANNA GREENBERG of this Laboratory; see also NACHMANSOHN[18]). At lower concentrations (10^{-6} *M* and lower) such lipid-soluble quaternary nitrogen ions as, *e.g.*, PAD and PDI show specific interactions with the acetylcholine system. These and related specific, directed attractions are responsible for the powerful reactivating action of PAM on inhibited acetylcholinesterase.

ACKNOWLEDGEMENTS

This investigation was supported by the Division of Research Grants and Fellowships, National Institutes of Health, Grant No. H-740, U.S. Public Health Service. The author is also indebted to Prof. D. NACHMANSOHN for his continuing advice and interest.

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A MAGNETO-KINETIC STUDY OF THE REACTION BETWEEN FERRIMYOGLOBIN AND METHYL HYDROGEN PEROXIDE

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(Received August 26th, 1959)

SUMMARY

1. The reaction between ferrimyoglobin and methyl hydrogen peroxide has been studied with a new instrument for measuring rapid changes in the magnetic susceptibility of dilute aqueous solutions of proteins.

2. A comparison of the magnetically and spectrophotometrically obtained rate data shows differences, increasing toward lower temperatures, which can be explained in part by the production of free radicals.

3. The molar susceptibility at 20° C of the myoglobin compound formed is $3300 \cdot 10^{-6}$ emu with a standard error of $500 \cdot 10^{-6}$ emu.

INTRODUCTION

The reactions of the hemoproteins with peroxides are of interest in themselves and pertinent to the elucidation of the mechanisms of peroxidase and catalase action. They have been studied spectrophotometrically and magnetometrically for many years. The recent development of rapid spectrophotometric techniques[1] has permitted observation of the kinetics of formation and decay of labile intermediates as well as the formation of stable compounds, and hence permitted the determination of the absorption spectra of the short-lived compounds. CHANCE suggested that a Rankine-balance type magnetic susceptometer be combined with a flow system to achieve great time resolution in magnetic susceptibility measurements, so that the magnetic moment of such labile intermediates could be determined. A most sensitive magnetic susceptometer of this nature has been developed and is described eleswhere[2]. Fig. 1 shows the performance of this instrument. Fig. 2 depicts a flow experiment where the magnetic susceptibility of the reaction mixture in half-cell 2 (which is constant in time for the constant flow rates Q_M and Q_S) is compared with the magnetic susceptibility of the unreacted solution in half-cell 1. While flow is in progress, steady-state reaction mixtures are observed with a time resolution of 0.05 sec or less, corresponding to the time spent by the mixture in the neighborhood of the magnet. When flow is stopped, the magnetic changes in the reaction mixture, now stationary in the cell, are followed with a time resolution corresponding to the electronically adjustable response of the magnet. In the experiments to be described, the response was somewhat faster than in Fig. 1A; for the fastest reaction the undamped angular frequency was 2 rad sec^{-1} and the ratio of damping to critical damping was 0.55.

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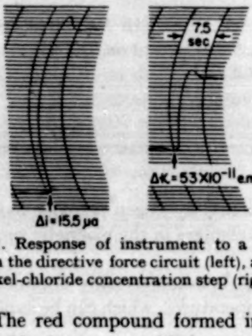

Fig. 1. Response of instrument to a current step in the directive force circuit (left), and to a nickel-chloride concentration step (right)[*].

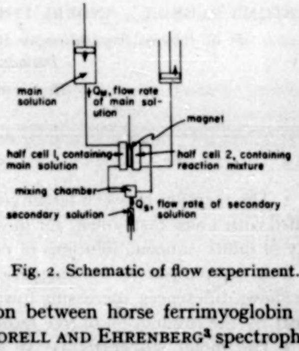
main solution
Q_M, flow rate of main solution
magnet
half cell 1, containing main solution
half cell 2, containing reaction mixture
mixing chamber
Q_S, flow rate of secondary solution
secondary solution

Fig. 2. Schematic of flow experiment.

The red compound formed in the reaction between horse ferrimyoglobin and methyl hydrogen peroxide was studied by THEORELL AND EHRENBERG[3] spectrophotometrically, using a Beckman apparatus, and magnetometrically, using their special Gouy-type balance[4]. Because of the instability of the compound at the ferrimyoglobin concentration of 650 μM required for the Gouy susceptometer, some of the compound had reverted to ferrimyoglobin during the relatively long time required to make a measurement with this instrument. Correcting for the amount of ferrimyoglobin present on the basis of spectrophotometric measurements, they obtained for the molar paramagnetic susceptibility of the compound a tentative value of $3000 \cdot 10^{-6}$ emu. They also measured the molar paramagnetic susceptibility of the compound formed in the reaction between ferrimyoglobin and H_2O_2, obtaining $3500 \cdot 10^{-6}$ emu. Recent calculations of GRIFFITH[5] show that a paramagnetic correction to the diamagnetism of the comparison hemoproteins should be made. Accordingly, these values are revised to 3300 and $3800 \cdot 10^{-6}$ emu.

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[*] From this Figure it can be seen that the poor time resolution of the original flow system (see Fig. 14 of ref.[2]) has been remedied by the introduction of a cell of improved design.

In an extensive series of experiments, George and Irvine[6–10] have studied the reactions of ferrimyoglobin with hydrogen peroxide, methyl and ethyl hydrogen peroxides, and other strong oxidizing agents. They have shown that all the myoglobin compounds formed have the same absorption spectrum and state of oxidation, one equivalent higher than ferrimyoglobin. They have suggested that there is only one compound and that it contains iron in the quadrivalent state. Additionally they have shown that when the peroxides, which have two oxidizing equivalents, react with ferrimyoglobin, a transient oxidizing entity is produced which they suggest is a free radical.

More recently Gibson, Ingram and Nicholls[11], utilizing electron spin resonance, have demonstrated the appearance of a free radical in the frozen reaction mixture of ferrimyoglobin and hydrogen peroxide. Their evidence indicates that the free radical is a product of a reaction of the transient oxidant rather than the peroxide, and is most likely situated in the globin where it is stabilized at the low temperature (90°K) required for the experiments. Additionally, in experiments with the magnetic field set for a g-value of 6, they find a quenching of the five unpaired electron signal of ferrimyoglobin when the peroxide compound is formed, which implies a small magnetic moment for the compound.

EXPERIMENTAL METHODS AND ANALYSIS OF DATA

The ferrimyoglobin, methyl hydrogen peroxide reaction has now been studied with the new rapid susceptometer. For reasons of temperature control, the flow scheme of Fig. 2 was used in these experiments so that the unreacted ferrimyoglobin was in one half-cell, the reaction mixture in the second half-cell, and the difference in the magnetic susceptibilities was measured. Table I is a compilation of the experimental conditions and the data obtained. The ferrimyoglobin concentrations were measured in terms of hemin by the pyridine hemochromogen method[12]. Some hemin is destroyed in this reaction (see DISCUSSION). The concentrations given are for unreacted ferrimyoglobin solutions. The methyl hydrogen peroxide was kindly supplied by A. C. Maehly[*]. The hydrogen peroxide content, determined by the pertitanic acid complex

TABLE I

EXPERIMENTAL CONDITIONS AND DATA OBTAINED

Expt.	*1*	*2*	*3*	*4*	*5*
[Ferrimyoglobin] × 10^6, M	45.3	18.5	25.2	26.9	23.6
[Me OOH] × 10^6, M	2300	210	240	200	190
pH[*]	8.36	8.23	8.25	8.29	8.28
Temperature °C	25	26	21	13.9	25.2
$\Delta\kappa \times 10^{11}$, emu (volume magnetic suscept. change)	—46.1 (mean of 4)	—21.1	—20.5 (mean of 2)	—	—27.9
k (rate constant), $\sec^{-1} M^{-1}$	—	—	370	210	1030

[*] In all experiments 0.10 M borate buffer was used.

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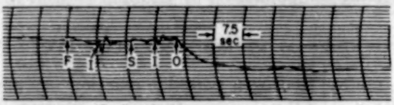
[*] The authors are pleased to thank Dr. Maehly for his generous help.

method, was 6 %. A correction was made for the O.D. of this impurity, and the concentration of the methyl hydrogen peroxide was measured spectrophotometrically at 230 mμ using an extinction coefficient of 32 M^{-1} cm^{-1}. The change in magnetic susceptibility obtained in experiment 4 is considered less reliable than other data and has not been included. The changes shown for expts. 1 and 3 are the mean values from identical reactions and carry weights of 4 and 2 respectively. The method of obtaining the rate constants is described in the paragraph on spectrophotometric experiments. Expts. 1 and 2 were done in 1955 and 1957 using an early cell with a time resolution of several seconds. Therefore, the kinetic data from these experiments are not included.

The record of expt. 5 is shown in Fig. 3. Methyl hydrogen peroxide was injected twice. The biphasic transients accompanying the injections (I) are due to jarring of the instrument when the stopcock was turned. The stopcock was turned off (S) with less disturbance. The steady-state reaction mixture in the cell during these injections corresponds to 0.10 sec after the beginning of the reaction. Essentially no deflection has occurred at this early stage. During the second injection period, the flow is stopped and the magnetic changes accompanying the remainder of the reaction are followed.



F flow begins
I injection of MeOOH
S injection stopped
O flow off

Fig. 3. Record of expt. 5.

The molar paramagnetic susceptibility of the compound formed in the reaction with methyl hydrogen peroxide is assumed to be independent of the pH because the absorption spectrum is pH independent[7]. The susceptibility is calculated from

$$\chi_{\text{compound}} = \chi_{\text{unreacted}} + \Delta\chi$$

where $\Delta\chi = 10^3 \Delta\kappa_{\text{final}}/[\text{ferrimyoglobin}]$ is the measured change in molar susceptibility.

The unreacted ferrimyoglobin exists in two forms, a brown neutral form with a molar paramagnetic susceptibility at 20° of $\chi_{\text{Fe}} = 13{,}980 \cdot 10^{-6}$ emu and a red alkaline form with a susceptibility of $\chi_{\text{FeOH}} = 11{,}330 \cdot 10^{-6}$ emu[13]. (These values have been revised according to the calculation of GRIFFITH[5]).

The molar susceptibility of the unreacted ferrimyoglobin solution is

$$\chi_{\text{unreacted}} = x\chi_{\text{Fe}} + (1 - x)\chi_{\text{FeOH}}$$

where the mole fraction x of the neutral form is given by

$$\log \frac{x}{1-x} = \text{p}K - \text{pH}$$

The temperature dependence of the pK has been determined[14]. After correcting the values for χ_{compound} to 20° by the Curie law, the mean value is calculated to be $3300 \cdot 10^{-6}$ emu with a standard error of $500 \cdot 10^{-6}$ emu.

At the high methyl hydrogen peroxide concentration used in the experiments, there was the possibility of a second reaction between the compound and unreacted MeOOH to form another compound, resulting in a mixture of compounds. This possibility was examined by recording the change in the absorption spectrum of ferrimyoglobin upon addition of MeOOH to make molar ratios of 1.5, 3.0, 4.5, and 7.5. The reactions were permitted to go to completion, and then the differential absorption spectrum was recorded from 500 mμ to 650 mμ. Confirming the observation of George and Irvine[7], the data show that a molar ratio somewhat greater than 3:1 is required for full formation of the compound at this pH. Within the accuracy of the spectrophotometer, the change in the absorption spectrum is the same for molar ratios of 4.5 and 7.5. At the molar ratios of 1.5 and 3.0 the change in the absorption spectrum is simply less intense. Therefore, only one spectrophotometrically identifiable compound is formed in this range of relative concentrations.

Spectrophotometric data were obtained on the kinetics of formation of the compound under the conditions of the magnetic expts. 3, 4 and 5. For the spectrophotometric studies, a split-beam recording spectrophotometer operated with a 0–67% rise time of 1 sec was used[15]. The time course of the reaction, which was followed at 550 mμ, was found not to obey second-order kinetics even when the effective excess oxidant was taken smaller than the nominal excess ratio, as might be suggested by the high molar ratio required for complete reaction. In the absence of full knowledge of the actual reaction mechanism, one can still define a rate constant as the rate of formation of compound per unit concentration of ferrimyoglobin and per unit concentration of oxidant. Since the concentrations are known only at the beginning of the reaction, and the initial 1.5 or 2 sec are obscured by a mixing artefact, it was necessary to extrapolate the time course of the observed compound concentration back to the initial point. For this purpose of extrapolation, some empirical descriptions of our data were tried. With a power-law dependence of the form

$$y = m_0\,[1 - (1 + t/T)^{-1/q}]$$

where y is the concentration of compound at the time t and m_0 is the initial ferrimyoglobin concentration, it was possible to adjust q and T to obtain an excellent fit. The second-order rate constant,

$$k = [\mathrm{Me\ OOH}]^{-1}\,(m_0 - y)^{-1}\,\mathrm{d}y/\mathrm{d}t,$$

is then given by

$$k = 1/qTa_0$$

where a_0 is the initial concentration of MeOOH. q determines the shape of the reaction curve, and will therefore depend upon the conditions of the reaction. For the spectrophotometrically observed reactions, the shape factor q is found to be the following function of the excess ratio a_0/m_0,

$$q = 8.3\,(a_0/m_0)^{-3/2}$$

The time courses of the magnetic susceptibilities fit the same empirical power function when q was calculated from the above formula and only T was adjusted. Thus there is no indication of different shape between the magnetic and spectrophotometric kinetic curves. However, the magnetic data do not allow of an accurate shape analysis. The rate constants for the magnetic curves are calculated in the same way as the spectrophotometric constants and are significantly smaller at lower temperatures.

This is shown in Fig. 4 which is an Arrhenius plot of the rate constants. Straight lines through the magnetic and spectrophotometric points were fitted by the method of least squares.

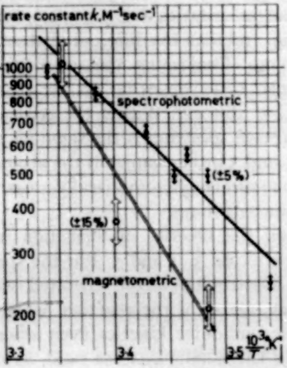


Fig. 4. Arrhenius plot of magnetic and Spectrophotometric rate data.

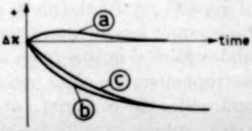


Fig. 5. *a*: the production and disappearance of free radicals; *b*: conversion of ferrimyoglobin to compound; *c*: observed change in magnetic susceptibility = *a* + *b*.

DISCUSSION

The existence of differences between the rates of change of optical density and magnetic susceptibility with time is consistent with the liberation of a free radical when ferrimyoglobin and methyl hydrogen peroxide react. This mechanism is one of two discussed by GEORGE AND IRVINE for the reaction between ferrimyoglobin and hydrogen peroxide[10]. It is interesting to note that the latter reaction proceeds about 5 times more slowly but has the same activation energy of 14,000 cal as calculated from the spectrophotometric data. The magnetically determined rate constants would not be expected to obey an Arrhenius relation if two or more magnetic processes contribute to the observed kinetic curve. The effect of the production and disappearance of free radical upon the time course of the magnetic susceptibility is shown schematically in Fig. 5. The observed change proceeds at a slower rate than the rate of conversion of ferrimyoglobin to compound alone. Spectrophotometrically only this conversion is followed (except possibly for a small effect due to the degradation of myoglobin;—see below). Because of the factor of eight between the molar magnetic susceptibility change in the myoglobin and the much smaller molar susceptibility of free radicals, it is difficult to account for the differences in the spectrophotometrically and magnetometrically determined rates on this basis alone. However, there is another observation which bears upon the problem.

Pyridine hemochromogen determinations of hemin concentration before and after the experiments show that as much as 11 % of the hemin of the myoglobin is destroyed during the reaction. The nature of the breakdown products has not yet been investigated so that their contributions to the magnetic susceptibility and to the O.D. are not quantitatively known but are almost certainly observable. It is very likely that the rate at which the degradation products are formed has an influence upon the spectrophotometric and magnetometric kinetic curves.

The value obtained for the molar susceptibility of the compound does not contain contributions from free radicals since these will have reacted to form stable diamagnetic products by the end of a magnetic run. However, the breakdown products will contribute to the extent that their molar susceptibilities differ from that of the compound. The amount, and possibly the types of breakdown product as well, depend upon the experimental conditions, and this variability may account for part of the spread in the values obtained for the molar susceptibility of the compound. The mean value of $3300 \cdot 10^{-6}$ emu at 20° is that for a complex of unit spin (two unpaired electrons). a spin which is theoretically possible for the d-shell configuration of GEORGE's postulated quadrivalent iron. It must be realized that for the small temperature range over which the magnetic experiments were made, the data are not sufficiently precise to test whether or not there is the Curie law dependence appropriate to a complex of unit spin. The molar susceptibility does not differ from the values obtained earlier by THEORELL AND EHRENBERG for both the hydrogen peroxide and methyl hydrogen peroxide reactions and supports the contention of GEORGE AND IRVINE that the same compound is formed in both reactions.

It is advisable now to extend the range of temperature and to investigate the breakdown products so that their effects upon the data can be evaluated. It would also be most useful to obtain data on the time course of the oxidant concentration. Until single large crystals of the compound are available, it is not likely that paramagnetic resonance will be of further help in elaborating the configuration of the d-shell.

ACKNOWLEDGEMENTS

The authors wish to thank F. A. MULLER for his assistance in analysing the data, and B. CHANCE, P. GEORGE Aand D. H. IRVINE for stimulating and helpful discussions.

This investigation was supported in its early stages by a Predoctoral Research Fellowship to A. S. BRILL from the Division of Research Grants of the United States Public Health Service.

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PURIFICATION OF PLACENTAL ALKALINE PHOSPHATASE

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(Recived August 10th, 1959)

SUMMARY

1. Alkaline phosphatase has been prepared in a highly-purified form from human placental tissue.

2. Two methods have been used for extracting the enzyme, *i.e.* the butanol extraction procedure of MORTON and the acetone–toluene–ethylacetate autolysis procedure of ALBERS.

3. The specific activity of alkaline phosphatase prepared by the butanol extraction method was 1150 KING-ARMSTRONG units/mg N, and that prepared by the autolysis method was 990 units.

4. The highly concentrated alkaline phosphatase was further purified by paper electrophoresis. The resulting enzyme preparation had a phosphatase activity of 1750 KING-ARMSTRONG units/mg N. This was less than was calculated to be present on the paper electrophoretic strip. But it was comparable with the highest activities obtained by other workers, when converted to their units by calculation. Placental tissue thus seems to have yielded as highly active a phosphatase as other tissues previously studied.

5. An attempt to purify alkaline phosphatase with an ion exchange resin (Dowex-2) was not successful.

INTRODUCTION

The preparation and purification of alkaline phosphatase has been the subject of much investigation. Sources rich in the enzyme have usually been used, such as kidney, whole intestine, intestinal mucosa, bone (normal and rickitic), and faeces. The methods used vary from simple water extraction followed by precipitation with ethanol and ether[1], to autolysis of the tissue with water and ethyl acetate[2] followed by fractionation with acetone[3] and (in other studies, ABUL-FADL AND KING[4]) by tryptic digestion and further fractionation. MORTON[5,6] introduced butanol extraction which he found to disrupt the lipoprotein, without denaturing the enzyme. He obtained a highly active preparation from both milk and calf intestinal mucosa. Preparation and concentration of placental alkaline phosphatase has been described in two previous papers[7,8]. Its further purification, by fractional precipitation and paper electrophoresis, is described here.

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EXPERIMENTAL

Collection and cleaning of placentas

The placentas were collected from the maternity ward as fresh as possible. The cord and membrane were removed, and the placenta freed from blood before clotting occurred: it was otherwise very difficult to get rid of clots and sometimes impossible. Perfusion with saline or saline containing heparin was of value only if the placenta was quite fresh. The best way to free the placenta of blood is to use a strong jet of water, cannulating one artery (or vein) after another until the whole organ is blanched. The cleaning of one placenta took about 1.5 h. Whenever it was not possible to begin the isolation and purification procedures immediately, the organ was stored at —15° after cleaning.

Preparation of aqueous placental extract

The placenta, cleaned as described above, was cut into small pieces, dried with blotting paper and weighed. It was then minced in a mechanical mincer, mixed with an equal volume of water and homogenized in a waring blender for 2 min. The homogenate was divided into 4 equal parts; one was left untreated, to the second an equal volume of butanol was added, the third was treated with 10 % of its volume of toluene, and to the fourth was added 10 % of its volume of toluene–ethyl acetate (1:1). All were stirred continuously for 30 min at room temperature. A sample of each mixture was centrifuged at 2000 rev./min for 15 min. The enzyme activity was determined in the aqueous layers at different pH values, using the phenyl phosphate method of KIND AND KING[9] (amino-antipyrine–ferricyanide) and expressing the result as KING-ARMSTRONG[10] (K-A)units, *i.e.* mg phenol liberated in 15 min.

The remainder of each of the mixtures was allowed to stand for 24 h at room temperature, and the enzyme activity then determined at the optimum alkaline pH. (The acid phosphatase was destroyed in all the mixtures except that containing only water.) Liberation of alkaline phosphatase in the solution was best with butanol, whether the tests were performed immediately or after 24 h. Results are shown in Table I.

Other organic solvents were compared with butanol for their efficiency in

TABLE I

ENZYME ACTIVITY AT DIFFERENT pH VALUES OF PLACENTAL EXTRACTS PREPARED BY DIFFERENT METHODS

Enzyme	*Enzyme activity in* KING-ARMSTRONG *units/g wet tissue in extracts separated at 30 min (and at 24 h)*									
	pH 3.6	*5*	*5.6*	*6.5*	*7.4*	*8.4*	*9.4*	*9.7*	*10*	*10.5*
Fresh water extract	0	3	2	0	5	7	35	48	60 (59)	57
Butanol extract	0	0	0	0	3	11	62	85	107 (105)	103
Toluene extract	0	0	0	0	1	6	34	44	56 (60)	54
Toluene + ethyl acetate extract	0	0	0	0	3	8	20	30	40 (50)	38

bringing alkaline phosphatase into solution. They are as follows, and the relative activities of their extract, compared with butanol, are given as numbers in parentheses: butanol (1), amyl alcohol (0.8), isopropyl ether (0.7), toluene (0.7), water alone (0.7), toluene + ethyl acetate (0.6), carbon tetrachloride (0.5), cychlohexanol (0.5), chloroform (0.4), propanol (0.2). The results emphasize the superiority of butanol.

Comparison of ALBERS' *autolysis method with* MORTON'S *butanol extraction method*

ALBERS' *autolysis method*

Extraction of enzyme: The mixed clean placentas were homogenized with an equal volume of water for 2 min. Pure acetone was added slowly with continuous stirring until a final concentration of 25 % was obtained. Toluene–ethyl acetate mixture (1:1) was then added (10 %). The whole mixture was thoroughly stirred for 1 h and then allowed to stand at room temperature for 3 days with occasional stirring. The mixture was squeezed through muslin, and centrifuged at 2000 rev./min for 30 min. If the supernatant solution was turbid, it was filtered through fluted filter paper. The specific phosphatase activity at this stage ranged from 9 to 13 K-A units/mg N. The clear aqueous solution showed no precipitate when adjusted to pH 4.9 with acetic acid. It was brought to pH 6.5 with NaOH.

Fractionation with acetone: The enzyme solution was chilled to —5°, and acetone at the same temperature added to a final concentration of 55 %. After 30 min at —5°, the solution was centrifuged and the precipitate washed 3 times with cold acetone and dried under vacuum over $CaCl_2$. The crude acetone powder was thus collected from 16 placentas. The specific activity of the powder was 80 K-A units/mg N. Attempts at further purification with acetone were not successful.

Ammonium sulphate fractionation: A 2 % solution of the enzyme powder was prepared, any insoluble material being removed by centrifugation. To the clear solution $(NH_4)_2SO_4$ powder (previously finely ground) was added until 0.4 saturation was obtained, the formula of KUNITZ[11] being used to calculate the amount of $(NH_4)_2SO_4$. The precipitate was centrifuged off and discarded. To the clear supernatant more $(NH_4)_2SO_4$ was added to raise its concentration to 0.5 saturation. The precipitate formed was separated by centrifugation, dissolved in water, and dialysed in a cellophane bag against running tap water overnight and then against frequent changes of distilled water. The specific activity of the enzyme solution was now 225 units/mg N.

Refractionation with acetone: The pH of the enzyme solution was adjusted to 6.2 with dilute acetic acid. The solution was chilled and acetone added to 35 %. The small precipitate was removed and more acetone added to 50 %. The precipitate was centrifuged, washed with chilled acetone and dried under vacuum over $CaCl_2$. This powder had a specific activity of 550 K-A units/mg N.

Refractionation with acetone between 35 and 45 % gave a preparation of 990 units/mg N. No further purification could be achieved by further refractionation with acetone.

MORTON'S *butanol method*

Conditions for butanol extraction: The effectiveness of extraction is influenced by the amount of butanol used: 1.5 ml/g is the minimum favourable amount. It is necessary to stir vigorously for at least half an hour in order to obtain the thorough emulsification necessary for an enzyme-rich aqueous layer. Warming the butanol emulsion to 35° for 5 min before centrifuging was found very effective in causing the colloidal particles to coalesce, so that a clear aqueous layer was obtained after centrifuging. This treatment did not affect the enzyme activity.

The optimum pH for both the extraction of the enzyme and for its maintenance was 6.5. In the presence of butanol the enzyme solution was stable in the cold room (+ 5°) for about 7 days. Inactive protein can best be precipitated at pH 4.9. Acetone precipitation of enzyme-rich material is best carried out at —5° at pH 6.5.

Preparation of enzyme extract with butanol: One or more placentas were cleaned, weighed, cut into small pieces, minced and homogenized with an equal volume of water for 2 min. The pH was adjusted to 6.5 with *N* acetic acid. Butanol (1.5 ml/g placenta) was added gradually with continuous vigorous mechanical stirring for at least 30 min. The homogenate became cream-coloured and well emulsified. It was squeezed through muslin, and heated in a water bath to 35° for 5 min, when it was poured quickly into previously cooled 250 ml cups and centrifuged for 45 min at 1800 rev./min at —4°. The mixture separated into four layers, *i.e.* a heavy precipitate, a clear yellowish aqueous zone, over-laid by light particles, and a surface layer of excess butanol containing dissolved material. The aqueous layer was sucked off carefully, but it was not usually obtained free from some suspended material. It was therefore filtered through a thick layer of Hyflo supercel on Whatman No. 1 filter paper on 100 ml Buchner funnels. The filtration did not affect the enzyme activity, which was 15 K-A units/mg N at this stage.

Acidification of aqueous extract: The solution was brought to pH 4.9 by the careful addition of dilute acetic acid. It turned cloudy and was left at room temperature for 30 min to allow for complete precipitation of inactive material. The precipitate was removed by filtration. (There was no loss of enzyme activity during filtration.) The clear filtrate was adjusted to pH 6.5 with 0.1 *N* NaOH. The specific activity was now 40 K-A units/mg N.

Fractional precipitation with acetone: The enzyme solution was cooled to —1° and chilled acetone (—5°) was added carefully with continuous stirring to 30%, maintaining the temperature at —0°. After 1 h at —5° the solution was centrifuged at 1800 rev./min for 20 min. The supernatant (still at —5°) was treated with more chilled acetone to 40%. The precipitate was allowed to settle and then centrifuged, and washed thrice with chilled acetone. The powder was dried *in vacuo* over $CaCl_2$, and stored at —15°. Several batches of placentas, 36 in all, were worked up in this way. The pooled acetone-dried powder had a specific activity of 135 K-A units/mg N. Further fractionation with acetone was unprofitable at this stage.

Fractional precipitation with ammonium sulphate: A 2% solution of the enzyme powder was prepared, any insoluble material being removed by centrifugation. Powdered $(NH_4)_2SO_4$ was added to 0.4 saturation, a precipitate removed and discarded and more $(NH_4)_2SO_4$ added to 0.5 saturation. The precipitate was separated by centrifugation, dissolved in water, and dialysed in a cellophane bag against running tap water overnight and then against distilled water. The specific activity of the enzyme solution was 475 units/mg N.

Refractionation with acetone: The enzyme solution was adjusted with 0.05 *N* NaOH to pH 6.2, cooled and treated with acetone. The fraction obtained between

TABLE II

ENZYME ACTIVITY AT DIFFERENT STEPS OF PURIFICATION OF PLACENTAL ALKALINE PHOSPHATASE

Procedure	*Total units* × 10³*	*Units*/mg N*	*Yield (%)*
ALBERS' *autolysis method*			
Original homogenate		1	
Aqueous extract	90	3	100
Filtered autolysate	75	10	83
Acetone fractionation	70	80	78
Ammonium sulphate fractionation 0.4–0.5	35	225	39
Acetone fractionation (35–50 %)	20	550	22
Acetone refractionation (35–45 %)	12	990	13
MORTON's *butanol method*			
Original homogenate		1	
Aqueous extract	190	3	100
Butanol aqueous layer	170	16	89
Butanol aqueous layer after acidification	165	40	87
Acetone fractionation (30–40%)	150	135	79
Ammonium sulphate fractionation 0.4–0.5	80	475	42
Acetone refractionation (35–45%)	35	1150	18

* Units = KING-ARMSTRONG units.

35 and 45 % was collected, washed carefully thrice with cold acetone and dried *in vacuo* over $CaCl_2$. The specific activity of the powder was now 1150 K-A units/mg N. Further refractionation did not increase the specific activity; nor did treatment with activated charcoal.

Table II shows the results of the attempted purification by both the ALBERS and MORTON methods.

Purification by paper electrophoresis

LEVY AND MAZIA[12] achieved a three-fold increase in activity of renal alkaline phosphatase by paper electrophoresis. Elongated tracks were obtained, and the enzyme was only partially separated from accompanying protein. ROCHE AND BOUCHILLOUX[13] electrophoresed intestinal phosphatase, which separated into three components only one of which was active. BAMANN AND TIETZ[14] employed continuous electrophoresis successfully for the separation of lipase.

Apparatus: The electrophoresis boxes used were similar to those described by GRASSMAN AND HANNIG[15] and by TISELIUS AND FLODIN[16]. The filter paper strips were of Whatman No. 1, and were 28 × 4 cm.

Reagents: The buffer was phosphate of pH 8.0. The staining solution was 1 % lissamine green in 15 % acetic acid. The washing solution was 2 % acetic acid. For oiling, a mixture was made of 34 ml liquid paraffin, 17 ml bromonaphthalene, 20 ml xylene and 1 ml sorbitol monooleate. In order to reveal the presence of the alkaline phosphatase on the filter paper, a spraying solution was made of 0.01 *M* *p*-nitrophenyl phosphate in 0.1 *M* sodium carbonate–bicarbonate buffer[17] (1:1) of pH 9.9. This solution was prepared in small amounts and kept in the refrigerator.

Method: The enzyme preparation of specific activity 1150 K-A units/mg N was used. 50 mg were dissolved in 1 ml distilled water. The filter paper was immersed in the buffer, spread over a glass sheet and blotted lightly with dry filter paper, and then applied to the holder. 0.02 ml of the enzyme solution was run on to the edge of a glass slide, which was then applied across the strip about 9 cm from the cathodic end. A second strip was similarly treated, and the holder with its two strips placed in the apparatus. Care was taken that the horizontal part of the filter paper was well stretched and that the vertical parts dipped in the buffers were of equal length.

Six boxes were used at a time, thus running 12 strips simultaneously. After 20 min to attain equilibration, the current was switched on and maintained for 16 h (*i.e.* overnight).

One strip was dried at 95° for 20 min, stained with lissamine green for 15 min, washed in 2 % acetic acid until excess dye was removed, and finally dried at 95° for 10 min. It was then oiled and allowed to stand upright on a filter paper sheet to drain. After 20 min it was scanned in the automatic recording densitometer of LAURENCE[18] using a narrow-cut red light filter (Ilford 205) combined with a neutral filter of density 0.5 (*cf.* Fig. 2).

Another strip was left at room temperature until almost dry and then in a 37° oven until quite dry. It was sprayed lightly with *p*-nitrophenyl phosphate solution. A yellow band appeared almost immediately, due to the *p*-nitrophenol liberated at the site where the phosphatase activity resided. Having thus identified the segment of the filter paper on which the phosphatase activity appeared, the corresponding segments in the remaining ten sheets were cut out and collected. They were stored in a jar in the deep freeze. The operation was repeated with twelve more papers.

Elution: A Whatman No. 1 filter paper (28 × 4 cm) was folded double, and the closed end trimmed so as to form a blunt wedge, a triangle (1.5 × 3–4 cm) being cut from either side of the folded end (see Fig. 1*). The effect of this was to leave a blunt

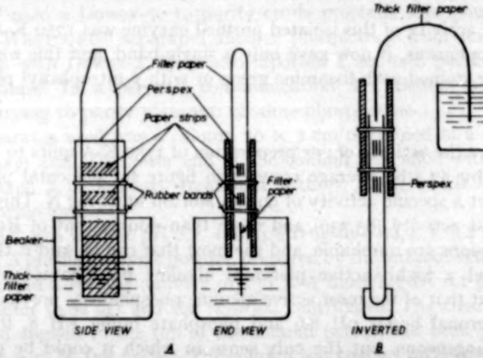

Fig. 1. Elution of phosphatase from segments of electrophoretic filter-paper. A, elution from segments into tip of folded wedge-shaped paper strip; B, elution from tip of the wedge into water.

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* The apparatus used to elute the electrophoretic strips was a modification of one used by our colleague, Dr. J. L. REIS, to whom we are grateful. It was found desirable to use a folded tip because a large amount of material could then be transferred during one operation. A single tip proved to be too thin and insufficiently rigid. Drying of the tip would occur if the transfer were carried out at room temperature, but was avoided by conducting the operation in the refrigerator. This apparatus and procedure are very efficient for dealing with small volumes of solution, and nearly 100 % recoveries can be obtained.

tip of 1 cm in width. The segments of filter paper (22) containing the enzymically active material were placed between the fold in two adjacent lots, about one-third of the distance from the open end of the folded strip. A thick filter paper strip was now placed in contact and in between the folded filter strip. The other end of the thick filter paper was intended to dip into a vessel containing distilled water. A strip of transparent plastic (perspex, 8 cm × 4.5 cm), was placed on each side of the above arrangement, and the two held together by two stout rubber bands. The assembly was held in a upright position, the thick paper dipping into a beaker of distilled water, with the ends of the two plastic strips just clearing its surface. The wedge-shaped upper end of the folded strip, projecting in a vertical position, slowly became saturated with the enzymically most active material, which was eluted from the 22 pieces of filter paper used for electrophoresis contained between the folded strip and which passed slowly up the strip by osmosis to concentrate in its tip. The whole procedure was carried out in the cold room overnight. After 16 h the assembly was inverted, the filter paper wick bent over to dip into a fresh beaker of water, and another small beaker without water placed below the wedge-shaped end of the folded strip, now in the bottom position. The enzymically active material in the tip now eluted into the small bottom beaker, as water passed slowly up over and down the thick paper strip, through the folded paper to its tip and dripped into the small beaker. The volume of the eluate was usually about 1 ml.

Dialysis

The eluate contained besides the enzyme a considerable amount of buffer salts. These were eliminated by dialysing the eluate against distilled water in the refrigerator overnight. The cellophane bag containing the eluate was filled so as to contain no vacant space.

The specific activity of this isolated purified enzyme was 1750 K-A units/mg N. On paper electrophoresis, it now gave only a single band, and this was in the same position whether stained with lissamine green or with *p*-nitrophenyl phosphate.

Activity

If we convert the activity of our preparation of 1750 K-A units to MORTON units by multiplying by 57 (the average conversion figure for placental phosphatase in our hands) we get a specific activity of 99,750 Morton units/mg N. This is more than MORTON's highest activity (83,530) and more than double that of ROCHE *et al.*[3,19]. But such conversions are unreliable, and the most that can be said is that we appear to have obtained a highly-active placental alkaline phosphatase whose activity seems to be about that of the most active alkaline phosphatases previously prepared. When run in veronal buffer pH 8.6 and phosphate buffer pH 8, it was electrophoretically homogeneous, but the only sense in which it could be claimed to be "pure" is that it seemed to be free of extraneous protein.

Fig. 2 shows the proportion of the total, before elution, which was enzymically active, *i.e.* that which stained yellow with *p*-nitrophenyl phosphate. It was 22 % of the whole lissamine green-stained protein area. In another preparation it was 25 %. If it be assumed that this area corresponds to the pure enzyme, then it would have a specific activity of about 4,600 units/mg N, since the enzyme solution used in the electrophoresis had a specific activity of 1,150 units. The specific activity of the eluted

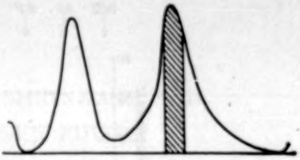

Fig. 2. Tracing of electrophoretic filter paper strip of purified phosphatase (1150 units/mg N) before elution. ———, total protein stained with lissamine-green; hatched area, phosphatase stained with *p*-nitrophenyl phosphate.

purified enzyme (1,750 units) was therefore much less than the amount conjectured from the assumption that the shaded area represented pure phosphatase. The discrepancy could be due to the conjecture being wrong or to a loss of enzyme activity in the elution and dialysis processes. Exposure of the enzyme to an alkaline pH for 16 h at room temperature may well have destroyed some of it. There may also have been a loss during the elution and dialysis, though these were carried out in the refrigerator and at approximate neutrality. Also, there may have been a loss of some co-factor, though there is no indication of this (compare ref. 8). Similar losses were experienced by ROCHE AND BOUCHILLOUX[13] who found that the specific activity of the active protein which they eluted after electrophoresis of intestinal phosphatase was less than the calculated one. CARLSON[20] used a vertical starch column for the electrophoretic purification of alkaline phosphatase. He found all the enzyme activity in a moving protein component which was 32 % of the total protein. This should have given him a 3-fold enrichment of the enzyme, but his isolated phosphatase was only twice as active. Continuous electrophoresis might be a more profitable procedure to obtain further purification of alkaline phosphatase.

Ion exchange

BOMAN[21] used a Dowex-50 to purify crude prostatic acid phosphatase 60-fold. BOMAN AND WESTLUND[22] separated alkaline phosphatase from acid phosphatase in human serum with Dowex-2. They also reported a 200-fold purification of red cell acid phosphatase. In a personal communication, Dr. BOMAN advised us to use Dowex-2 in trying to purify placental alkaline phosphatase.

The apparatus used was a column 20 × 2 cm contained in a glass tube with a tap at its lower end. The lower end of the column was filled with glass wool. The column received a preliminary treatment with *N* NaCl, which served to convert it to the chloride form.

Tris buffer was used at pH 7.2, and at strengths 0.02, 0.1, 0.2 and 0.4 *M*.

Method: A purified enzyme powder, prepared by the butanol extraction procedure, was used. It had a specific activity of 130 K-A units/mg N. 0.1 g was dissolved in 2 ml Tris buffer (0.02 *M*) and the solution centrifuged. The clear supernatant was collected and applied to the top of the column (which had been previously washed through with 0.02 *M* Tris buffer). The column was treated successively with step-wise increases of molarity of buffer solution (0.02, 0.1, 02., 0.4 and 1 *M*). Fractions were collected every half-hour with an automatic fraction collector. The volume of each fraction was about 2.5 ml. The enzyme activity was determined in each fraction, and the total amount of protein by measuring the extinction at 280 mμ in 1-cm cells in a Beckman DU spectrophotometer.

Result: Fig. 3 shows the results obtained. One curve represents the phosphatase

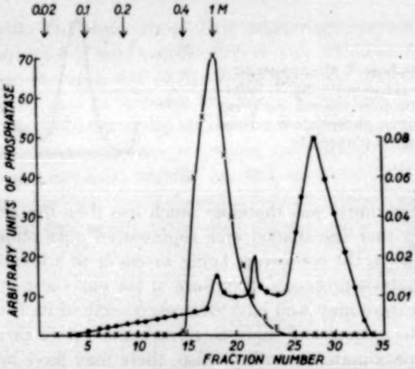

Fig. 3. Ion exchange separation of phosphatase. ×—×, Phosphatase; ●—●, Protein. Elution with 0.02–1 *M* Tris buffer.

activity, the other the extinction at 280 mμ. Most of the enzyme was eluted with the 0.4 *M* buffer. The extinction values showed that there are at least two inactive peaks in the solution. The recovery of the enzyme was about 50 %, and its specific activity increased from an initial 130 units to 230 units/mg N. The protein area under the phosphatase activity peak is about 23 % of the total. So one would expect a specific activity of about 570 units/mg N. Some loss of activity appears, therefore, to have taken place, as in the case of paper electrophoresis.

A further experiment was carried out with the highly-purified enzyme of 1,150 units/mg N, but no further increase of activity could be obtained.

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A STUDY OF THE CHEMICAL SHRINKAGE AND RELAXATION OF COLLAGEN FIBERS

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(Received August 31st, 1959)

SUMMARY

The chemical contraction and relaxation of collagen fibres are described in terms of chemical kinetics, and the factors influencing the course of fibre length variations are studied. The course of chemical contraction and relaxation is investigated both by measuring the fibre length and by determining the concentration of the liberated substances. The influence of the pH of the medium and that of reducing agents upon the shape of the curves showing the dependence of fibre length on time is studied.

INTRODUCTION

Though the thermal contraction and relaxation of collagen fibres have been thoroughly investigated[1], comparatively little attention has been paid to the so-called chemical contraction and relaxation[2-4]. The majority of papers[1,5] in this field has been restricted to the study of the influence of electrolytes and non-electrolytes on the shrinkage temperature of the collagen fibre, and in this connection it has been pointed out that perchlorate, thiocyanate and iodide ions lower the shrinkage temperature markedly.

BANGA, BALÓ AND SZABÓ[3-4] studied the changes of collagen fibres in 40 % potassium iodide and showed that the shrinkage of the fibre is connected with the splitting-off of a mucoproteinic substance and that relaxation is due to the rupture of the linkage between procollagen and metacollagen. Of interest is the finding by these authors that reducing substances acting in acid medium effect "rejuvenation" of the reactivity of the fibres and that, inversely, oxidizing agents bring about ageing of collagenous structures. They further showed that the course of contraction and relaxation materially depends on the age of the organism.

In applying this method to the study of some problems of pathophysiological character, however, we were carrying out a fundamental investigation of the individual factors which could act in chemical contraction and relaxation, as well as giving a description of the actual course of fibre length variations in terms of chemical kinetics. After studying these principal questions we were further occupied with the problem of a possible "rejuvenation" of the reactivity of collagen fibres in the sense described by the Hungarian authors[3-4].

THEORETICAL

We shall try to find an expression describing the dependence of the total fibre length l on time for the case of chemical shrinkage and relaxation of the collagen fibre, the course of which together with the notation of the quantities involved is given in Fig. 1.

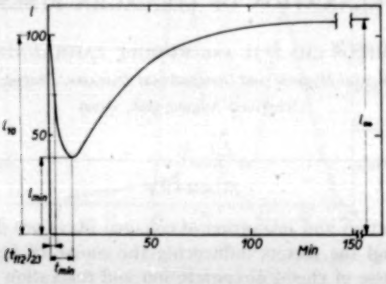

Fig. 1. Typical course of a model curve of chemical contraction and relaxation of collagen fibres. Nomenclature: l (fiber length), t (time), $l_{1,0}$ (fibre length at the time $t = 0$), l_{min} (minimum fibre length), l_∞ (extreme fibre length), $(t_{1/2})_{23}$ or t_{min} (time required for attainment of half the reduction of the initial fibre length or of maximum shrinkage respectively). Parameters of the curve: $k_{12} = 0.20$; $k_{23} = 2.00$; $k_{34} = 0.345$; $l_1 = 100$; $\varphi_2 = 1.0$; $\varphi_3 = 0.1$; $\varphi_4 = 1.083$.

Let us assume that the total length of the collagen fibre l at an arbitrary time is given by the sum of the lengths l_i of the fibre-forming components

$$l = \sum_i l_i \tag{1}$$

The minimum on the graph of the function (Fig. 1) as well as the relative positions of this minimum and the points of inflection indicate that at least three changes participate in the process of shrinkage and relaxation. From the shape of the graph of the function we conclude that it expresses a consecutive reaction, which can be described by the scheme

$$A_1 \xrightarrow{k_{12}} A_2 \xrightarrow{k_{23}} A_3 \xrightarrow{k_{34}} A_4 \tag{2}$$

We assume that the reactions in scheme (2) are irreversible reactions of the first order, which assumption appears to be justified with regard to experimental data.

The kinetic scheme (2) was studied by RAKOWSKI[6], who expressed the concentration of the components A_i as explicit functions of time.

Acting on the assumption that the "concentration" of the component A_i in the fibre under investigation is linearly proportional to its length l_i, we can represent the dependence of the length l_i ($i = 1, 2, 3, 4$) on time by

$$l_1 = l_{1,0} \exp(-k_{12}t) \tag{3}$$

$$l_2 = \varphi_2 \frac{l_{1,0}\, k_{12}}{k_{23} - k_{12}} (\exp[-k_{12}t] - \exp[-k_{23}t]) \tag{4}$$

$$l_3 = \varphi_3 l_{1,0} k_{12} k_{23} \left\{ \frac{\exp(-k_{12}t)}{(k_{23}-k_{12})(k_{34}-k_{12})} - \frac{\exp(-k_{23}t)}{(k_{23}-k_{12})(k_{34}-k_{23})} + \frac{\exp(-k_{34}t)}{(k_{34}-k_{23})(k_{34}-k_{12})} \right\} \tag{5}$$

$$l_4 = \varphi_4 l_{1,0} \left\{ 1 - \frac{k_{23}k_{34} \exp(-k_{12}t)}{(k_{23}-k_{12})(k_{34}-k_{12})} + \frac{k_{12}k_{34} \exp(-k_{23}t)}{(k_{23}-k_{12})(k_{34}-k_{23})} - \frac{k_{12}k_{23} \exp(-k_{34}t)}{(k_{34}-k_{23})(k_{34}-k_{12})} \right\} \tag{6}$$

In eqns. (3)–(6) k_{ij} denotes the first-order rate constant of the conversion of A_i to A_j, l_1 is the length of the component A_1 at time t, $l_{1,0}$ represents the length of the fibre A_1 at time $t = 0$, φ_i are the relative lengths of the components A_i, defined by

$$\varphi_i = l_{i,0}/l_{1,0} \ (i = 1,2,3,4) \tag{7}$$

The values $l_{i,0}$ ($i = 2, 3, 4$) denote the length of the fibre A_2, A_3, A_4, which is equivalent to the length of the fibre A_1 at time $t = 0$, hence $A_{1,0}$.

For the cases in which we are interested, the dimensionless parameters satisfy the inequalities

$$\varphi_2 \leqq 1 \tag{8}$$

$$0 < \varphi_3 < 1 \tag{9}$$

$$\varphi_4 \lesseqgtr 1 \tag{10}$$

The total fibre length l, accessible to measurement, is according to eqn. (1) given by the sum of eqns. (3)–(6). Thus we obtain eqn. (11)

$$l = \sum_{i=1}^{4} l_i = K_1 \exp(-k_{12}t) + K_2 \exp(-k_{23}t) + K_3 \exp(-k_{34}t) + l_{1,0}\varphi_4 \tag{11}$$

The constants K_i are defined by

$$K_1 = \frac{l_{1.0}}{(k_{23}-k_{12})(k_{34}-k_{12})}\{((k_{23}-k_{12})(k_{34}-k_{12}) + \varphi_2 k_{12}(k_{34}-k_{12}) + \varphi_3 k_{12}k_{23} - \varphi_4 k_{24}k_{34}\} \tag{12}$$

$$K_2 = \frac{l_{1,0}}{(k_{23}-k_{12})(k_{34}-k_{23})}\{-\varphi_2 k_{12}(k_{34}-k_{23}) - \varphi_3 k_{12}k_{23} + \varphi_4 k_{12}k_{34}\} \tag{13}$$

$$K_3 = \frac{l_{1.0}k_{12}k_{23}}{(k_{34}-k_{23})(k_{34}-k_{12})}\{\varphi_3 - \varphi_4\} \tag{14}$$

The magnitudes appearing in eqns. (12)–(14) have already been defined. From the first and second derivatives of the total length l (eqn. (11)) with respect to time we can calculate the conditions for the minimum and the point of inflection of the function. Practically, however, the respective relations are hardly usable because of the complexity of the summary constants they contain.

The determination of the rate constants k_{12}, k_{23} and k_{34} (eqn. (11)) from the known time-dependent course of shrinkage and relaxation is a very intricate task, admitting no exact solution at all. In the investigated cases, however, the rate constants k_{12} and k_{23} are always sufficiently large in comparison with the rate constant k_{34}. Therefore, it is possible for sufficiently large time values to neglect in equation (11) the terms involving the exponential curves $e^{-k_{12}t}$ and $e^{-k_{23}t}$ beside the other terms. Thus equation (15) is satisfied with sufficient accuracy:

$$l = K_3 \exp(-k_{34}t) + l_{1,0}\varphi_4 \tag{15}$$

By rearrangement and taking the logarithm of equation (15) we obtain

$$\lg(l - l_{1,0}\varphi_4) = \lg K_3 - k_{34}t \tag{16}$$

Equation (16) defines a straight line, the slope of which gives the rate constant k_{34}. Unfortunately, the exact expressions from which the rate constants k_{12} and k_{23} could be ascertained are too complicated. For this reason, we approximately replace the constant k_{23} by half the time $(t_{1/2})_{23}$ required for attainment of the minimum fibre length ($l_{\min}$), which is a value roughly inversely proportional to the constant k_{23} sought.

Finally, it should be noted that by means of the criterion suggested by VRIENS[7] for a reaction of the type $A_1 \longrightarrow A_2 \longrightarrow A_3$ one can be certain that the shrinkage and relaxation of collagen fibres proceed according to the scheme of a consecutive and not a side reaction. If, namely, the ratio of the concentration of the split-off constituent causing shrinkage to the concentration of the substance causing relaxation in the initial phase of the reaction is approximately constant, the scheme of side reactions predominates, and, inversely, if this ratio materially diminishes with time (which applies in our case), the scheme of the consecutive reaction becomes manifest.

Finally, it may be added that in special cases the three-stage scheme considered (2) becomes a two-stage scheme, and then it is a simple matter to determine the rate constants.

METHODS

The collagen fibres were isolated either from the tails of living, anaesthetized rats, or from tails cut off in such a way that the skin was left on the preparation and the fibre was pulled out through a cut of about 0.5 cm in length at a distance of 2 cm from the tail end. Since we did not always succeed in pulling out a single fibre, the extracted fibres were disintegrated as quickly as possible into elementary fibrils, that were further indivisable mechanically; after isolation these were immediately clamped into an indented rubber stopper. The free end of the fibre was at a certain distance pressed into a lead foil weighing 0.050 g, unless otherwise stated. The suspended fibre was transferred with the respective solution to a test tube of 14 × 1.4 cm, placed in a water thermostat maintaining the prescribed temperature with an accuracy of ± 0.1°. The experiments were carried out with collagen fibres from adult rats; the diameter of the fibres was from 0.14 to 0.15 mm.

Experimentally it was found that the error in determining the rate constant (k_{34}) under the usual conditions amounted to ± 10 %, the error in ascertaining the time required to attain the minimum length ($t_{\min}$) was about ± 15 %, and the deviations of the other magnitudes varied by about ± 5 %. Experiments with fibres of 2–10 cm in length showed that the uncertainty in fibre length manifesting itself in routine tests involves a negligible error in the determination of the quantity under investigation.

The influence of the magnitude of the weight (0.050–0.150 g) is apparent from Table I.

TABLE I

INFLUENCE OF THE MAGNITUDE OF THE LOAD (m) ON THE COURSE OF SHRINKAGE AND RELAXATION OF COLLAGEN FIBRES FROM A RAT WEIGHING 135 g

2.5 *M* potassium iodide, 24.0°, initial fibre length 6.0 cm.

m (g)	$l_{\min}$ (cm)	l_{∞} (cm)	$t_{\min}$ (min)	$10^2 \cdot k_{34}$ (min^{-1})	φ_4	$\varphi_{\min}$
0.050	3.0	5.4	3.0	5.0	0.90	0.50
0.100	3.1	5.8	3.5	4.7	0.97	0.52
0.150	3.5	6.55	3.3	3.3	1.09	0.58

RESULTS AND DISCUSSION

Measurement of total fibre length and of concentration of the split-off substances

In the investigation of the substances split-off in the course of shrinkage and relaxation of collagen fibres in 2.5 *M* $NaClO_4$ the experiment was carried out as follows. About 150 fibres, isolated by the above method, were transferred to a solution of 2.5 *M* $NaClO_4$ and in the course of shrinkage and relaxation specimens of the solution were withdrawn to measure their light absorption at 275 mμ (absorption corresponding to amino acids with aromatic nuclei in non-collagenous proteins), and after acid hydrolysis of these specimens (6 *N* HCl, 140°, 4 h in sealed test tubes) the hydroxyproline content was determined according to NEUMAN AND LOGAN[8] and that of proline according to CHINARD[9].

Fig. 2 illustrates the dependence of these quantities on time, the individual curves (1, 2, 3) being superimposed on the diagrams of the exponential functions. From this figure it is evident that a simple exponential curve covers the course of the experimental values for the time beginning at $t = 3$ min. During the first three minutes the reaction proceeds relatively very rapidly and evidently exponentially too (Fig. 3). On the course of the variations of the absorption (for $t > 3$ min) at 275 mμ (Curve 2, Fig. 2) is superimposed the exponential curve for the value of the rate constant $k_{23} = 2.26 \cdot 10^{-2}$ min^{-1}. On the course of the concentrations of

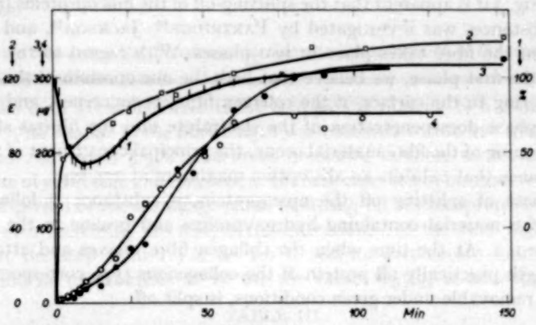

Fig. 2. Course of the reaction of the collagen fibre as observed by measurement of the total fibre length and of the concentration of the split-off substances. The total fibre length in cm (1), extinction of the substance with an absorption maximum at 275 mμ (2) and the concentrations of proline (3) and hydroxyproline (4) in the hydrolysates are plotted against time. At curve 1 the theoretical curve (eqn. (11)) is laid through experimental points for the constants: $k_{12} = 0.19$; $k_{23} = 1.9$; $k_{34} = 0.04$; $\psi_2 = 1.0$; $\psi_3 = 0.1$; $\varphi_4 = 1.083$. Data concerning the other curves are given in the text.

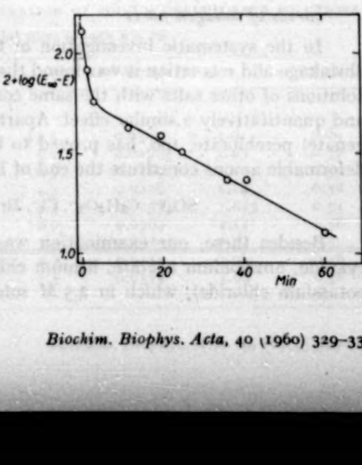

Fig. 3. Course of the splitting-off of the substance with an absorption maximum at 275 mμ plotted against time in semilogarithmic coordinates.

hydroxyproline (Curve 4, Fig. 2) and proline (Curve 3, Fig. 2) are superimposed empirical exponential curves.

Through experimentally ascertained points indicating the dependence of the total fibre length on time (Curve 1, Fig. 2) is laid a theoretical curve whose constants k_{34} and k_{23} were estimated from the course of the splitting-off of procollagen and mucoprotein, respectively. The value φ_4 necessary for the calculation was taken from experimental data on Curve 1; the values φ_2, φ_3 and k_{12} were determined by stepwise approximation.

On some fibres from the group which served for the experiment just described the course of the reaction was also studied by measuring the length, *i.e.* by the method currently used in this work. Curve 1 in Fig. 2 represents the course of shrinkage and relaxation; through experimentally ascertained points a theoretical curve is laid based on the rate constants resulting from the investigation of the concentration of proline and hydroxyproline and from the determination of the light absorption at 275 mμ (Fig. 2).

From the results obtained it follows that the fastest process in the chemical contraction and relaxation of the collagen fibre is the splitting-off of a substance exhibiting an absorption maximum at 275 mμ and containing no hydroxyproline. As far as time is concerned, the phase of the contraction of the collagen fibre corresponds to this reaction.

From Fig. 3 it is apparent that the splitting-off of the mucoproteins (the character of these substances was investigated by PARTRIDGE[10], JACKSON[11], and BANGA AND BALÓ[12]) from the fibre takes place in two phases. With regard to the considerable velocity of the first phase, we believe that here the mucoprotein of the ground substance, adhering to the surface of the collagen fibre, is concerned, and that only in the second phase does penetration of the electrolyte into the fibrous structure and gradual loosening of the fibre material occur; the principal constituent of this material is the substance that exhibits an absorption maximum at 275 mμ.

The phase of splitting off the mucoproteinous substance is followed by the liberation of a material containing hydroxyproline and proline in the ratio of approximately 1:1. At the time when the collagen fibre relaxes and attains its final extreme length practically all protein of the collagenous type corresponding to procollagen[3-4], removable under given conditions, is split off.

Influence of the composition of the solution upon the course of the shrinkage and relaxation curves of collagen fibres

In the systematic investigation of the anions and cations producing chemical shrinkage and relaxation it was found that, in addition to 2.5 *M* potassium iodide[2-4], solutions of other salts with the same concentration (Table II) exhibit qualitatively and quantitatively a similar effect. Apart from pseudo-halides (thiocyanate, xanthogenate) perchlorate, too, has proved to be effective. It is known that these highly deformable anions constitute the end of Hofmeister's series of lyotropic anions:

$$SO_4^{2-}, C_2H_3O_2^-, Cl^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-$$

Besides these, our examination was extended to a number of salts (sodium cyanide, ammonium acetate, lithium chloride, sodium bromide, potassium nitrate, potassium chloride), which in 2.5 *M* solution did not produce any changes in the

TABLE II

INFLUENCE OF THE ANION COMPOSITION ON SHRINKAGE AND RELAXATION OF COLLAGEN FIBRES FROM A RAT WEIGHING 150 g

2.5 *M* solutions of sodium and potassium salts, 24.0°, load 0.150 g, initial fibre length 6 cm.

Salt	l_{min} (cm)	l_{∞} (cm)	$(t_{1/2})_{23}$ (min)	t_{min} (min)	$10^3 \cdot k_{34}$ (min^{-1})	φ_4	φ_{min}
Sodium iodide	3.3	6.2_8	3.0	9.0	3.6	1.04	0.55
Potassium thiocyanate	3.3	6.8_7	1.0	2.0	2.8	1.14	0.55
Potassium ethylxanthogenate	2.25	8	0.5	2.0	5	1.3	0.38
Sodium perchlorate	3.0_7	6.4_8	2.0	5.5	2.5	1.08	0.51

length of collagen fibres within 180 min. Also ineffective were 5 *M* solutions of sodium chloride and sodium nitrate. Since sodium perchlorate is in the group of effective salts most stable towards oxidizing and reducing agents and most easily obtainable in a pure, defined state, most of the further experiments were carried out with this salt.

In the series of alkali iodides (from lithium up to caesium) we found that under the conditions described the cation exerts no influence on the curves of shrinkage and relaxation.

Influence of the concentration of sodium perchlorate solutions and of the ionic strength of the reaction medium

From the data given in Table III it is apparent that up to a concentration of 1.9 *M* $NaClO_4$ no change in the fibre length was observed within 120 min. At concentrations from 2 *M* up to 3.5 *M*, the fibres immersed in these solutions exhibit the typical course of shrinkage and relaxation. In these cases it can be observed (Table III) that the half-period of the shrinkage phase (splitting-off of mucoprotein) significantly diminishes with increasing electrolyte concentration. For the very high rise in the reactivity at the step from 1.9 *M* to 2.0 *M* sodium perchlorate solution we have hitherto found no explanation. If we use the values $(t_{1/2})_{23}$ as first approximations

TABLE III

DEPENDENCE OF THE TIME COURSE OF SHRINKAGE AND RELAXATION OF COLLAGEN FIBRES FROM A RAT WEIGHING 120 g ON THE CONCENTRATION OF SODIUM PERCHLORATE SOLUTIONS

25.0°, load 0.050 g, initial fibre length 6.0 cm.

M $NaClO_4$	l_{min} (cm)	l_{∞} (cm)	$(t_{1/2})_{23}$ (min)	t_{min} (min)	$10^3 \cdot k_{34}$ (min^{-1})	φ_4	φ_{min}
1.9	no changes within 120 min						
2.0	3.60	6.05	6	12.5	0.0385	1.01	0.60
2.1	3.55	6.20	4.5	3.0	0.02903	1.03	0.59
2.2	3.55	7.05	3.0	6.5	0.0289	1.17	0.59
2.3	3.50	6.85	2.0	4.5	0.0228	1.14	0.58
2.4	3.25	6.15	2.0	4.5	0.0253	1.025	0.54
2.5	3.50	6.65	3.0	8.0	0.0362	1.11	0.58
2.7_5	3.15	6.25	0.5	3.5	0.0325	1.04	0.52
3.0	2.85	6.2	1	3.5	0.0451	1.03	0.48
3.5	2.85	6.55	0.5	1.5	0.0322	1.09	0.48

for calculating the rate constants k_{23} of the splitting-off of mucoprotein and plot these rate constants against the molarity of sodium perchlorate, we obtain an approximately linear dependence. Hence it is evident that for the rate constant of the splitting-off of protein with an absorption maximum at 275 mμ, for a constant quantity of collagen fibres, eqn. (17) holds:

$$k_{23} = k\,[\mathrm{ClO_4^-}] \tag{17}$$

where $[\mathrm{ClO_4^-}]$ denotes the molarity of sodium perchlorate and k_{23} the corresponding rate constant. The suitability of this equation for the correlation of our data indicates that the splitting-off of mucoprotein (for $t > 3$ min) has the character of an ordinary chemical reaction.

Moreover, it was proved that a change in the ionic strength of the 2.5 M sodium perchlorate solution from the value of 2.5 M to 6.5 M (ionic strength adjusted with lithium chloride) does not bring about a significant change in the rate constant k_{34}. Changes of $(t_{1/2})_{23}$ are likewise not significant with regard to the experimental errors; only the magnitude of contraction rises with increasing ionic strength.

A striking feature in the evaluation of the influence of cations and anions upon the time-dependent course of shrinkage and relaxation of collagen fibres is the negligible effect of the composition of the cations in comparison with the pronounced effect of the anion composition. The effect of lithium, sodium and potassium iodides is practically the same. On the other hand, beginning with iodide, the anions forming the end of Hofmeister's series are effective. This relates to iodide, perchlorate, thiocyanate and xanthogenate. These relatively little solvated anions are known for their swelling effect. It may be assumed that shrinkage is due to a change in hydration and thus also to the character of the bonding forces of the protein molecules of the collagen fibre, as a result of the linking of the anions to these structures. With regard to the insignificant differences between the cations, it may further be concluded that the reaction causing shrinkage is probably due to sorption of the negatively charged, only slightly hydrated ion on the positively charged centre. On the whole, it can be stated that there are no significant differences in the efficiency of thiocyanate and perchlorate. Potassium ethyl xanthogenate is significantly more effective than other active anions in that both shrinkage and relaxation proceed essentially faster. However, we have observed that, in contrast to other reagents, the fibre ruptures before attaining its limiting length.

Dependence on temperature

In studying the dependence of shrinkage and relaxation on temperature we found that this dependence under the conditions applied, can be investigated only in a narrow temperature range. Excessive slowness or cessation of the process at lower temperatures and a very high rate at higher temperatures impede experimental investigation. This is obviously due to the comparatively high energy of activation of at least one of the rate processes.

As far as the relaxation process is concerned, we were able to study the dependence of the actual rate constants (k_{34}); the rate of the shrinkage process, on the other hand, we have characterized only by the value of the half-period $(t_{1/2})_{23}$, which, however, involves no change in the Arrhenius energy of activation (Table IV).

From the values of the energies of activation it is apparent that shrinkage in-

TABLE IV

ARRHENIUS ENERGY OF ACTIVATION OF THE SHRINKAGE AND RELAXATION PROCESS OF THE COLLAGEN FIBRE IN A 2.5 M SODIUM PERCHLORATE MEDIUM IN THE RANGE OF 16–30°

Process	*Rat weight (g)*	E^* *(kcal·mol⁻¹)*
Shrinkage	290	57 ± 4
Shrinkage	240	53 ± 4
Relaxation	290	9.6 ± 1

volves a process which, as is to be inferred from the high values of the energy of activation, is accompanied by the disappearance of relatively very firm chemical bonds. We know that this process splits off mucopolysaccharide, and perhaps mucoprotein, from the collagen fibre. On the other hand, the splitting-off of procollagen is a process that sets in much more readily. Since the corresponding value of the energy of activation amounts to only 10 kcal·mol^{-1}, we conclude that the splitting-off of procollagen is connected with the disappearance of comparatively weak bonds.

Influence of reducing agents and the pH value of the medium on the shrinkage and relaxation curves

BANGA, BALÓ AND SZABÓ[4,12] reported that by the action of various reducing agents in acid medium collagen fibres from adult or old rats assume the properties of fibres isolated from younger organisms. The magnitude of shrinkage is here smaller and rapid relaxation sets in, attaining a greater length than the initial one.

With regard to this finding, we have studied the influence of 0.01 M solutions of a number of reducing agents on the course of chemical contraction and relaxation of collagen fibres. The reducing substances used were quinol, chromium dichloride, titanium trichloride, ferrous ammonium sulphate and ascorbic acid. The last-named substance had to be employed also in solutions of 0.001 M concentration.

Freshly isolated collagen fibres from rat tails were soaked for 30 min in solutions of these substances, and it was found that no significant variation of the length sets in and that the general appearance of the fibre changes characteristically[13,14]. The fibre was then slightly dried with filter paper and immediately transferred to a 2.5 M sodium perchlorate solution, and the dependence of the length on time investigated.

The results obtained (Table V) are not in agreement with the findings of the Hungarian authors. Among five reducing substances examined only the 0.001 M solution of ascorbic acid brought about a pronounced change of the contractibility of the fibre in the sense of "rejuvenation". The other substances, apart from titanium trichloride, caused a marked change in the course of shrinkage and relaxation, which according to BANGA, BALÓ AND SZABÓ[4] could be characterized as "ageing". Soaking in 0.01 M ascorbic acid for 30 min resulted in distinct swelling of the fibre, which became rubber-like and elastic and immediately extended when loaded with a weight of 0.050 g. For this reason, we used concentrations lower by one order of magnitude (0.001 M), in which a certain amount of swelling still took place, but the fibre could be further investigated in a solution of sodium perchlorate.

In order to find out whether the "rejuvenating" effect of ascorbic acid lies in the reducing properties of this substance or is due to the acidity of its solution, we

TABLE V

INFLUENCE OF PRECEDING ACTION (30 min) OF AQUEOUS SOLUTIONS OF REDUCING SUBSTANCES ON THE COURSE OF SHRINKAGE AND RELAXATION

2.5 *M* sodium perchlorate, 25.0°, load 0.050 g, initial fibre length 6 cm.

Reducing substance (0.01 M solutions)	*pH*	*Rat weight (g)*	l_{min} *(cm)*	l_{∞} *(cm)*	$(t_{1/2})_{23}$ *(min)*	t_{min} *(min)*	$10^2 \cdot k_{34}$ *(min⁻¹)*	φ_4	φ_{min}
Control fiber	—	130	3.5	6.13	2.5	7	3.98	1.02	0.583
Quinol	7.1	130	2.6	4.48	3	9	1.36	0.74	0.434
Chromium dichloride	5.3	130	3.6_3	5.28	7	17.5	2.35	0.94	0.608
Titanium trichloride	6.1	130	3.1_5	5.93	2	5.5	4.33	0.99	0.525
Ferrous ammonium sulphate	6.3	130	3.2	5.78	2	8	3.98	0.96	0.533
Ascorbic acid	2.6	250	5.9			3			
Ascorbic acid*	3.3	250	1.8	6.0	1	8	1.27	1.00	0.300
Fysiological solution	7.2	250	2.8	6.2	3	7.5	2.30	1.04	0.466

* 0.001 *M* solution.

TABLE VI

INFLUENCE OF PRECEDING ACTION (30 min) OF BRITTON–ROBINSON BUFFER ON THE COURSE OF SHRINKAGE AND RELAXATION

2.5 *M* sodium perchlorate, 25.0°, load 0.050 g, initial fibre length 6.0 cm.

pH	l_{min} *(cm)*	l_{∞} *(cm)*	$(t_{1/2})_{23}$ *(min)*	t_{min} *(min)*	$10^2 \cdot k_{34}$ *(min⁻¹)*	φ_4	φ_{min}
3.95	6.0	6.7	0.0	0.0	70*	1.11	1.00
4.8	5.8	6.3	0.5	2.0	20*	1.05	0.97
5.2	5.7	6.6	1.0	2.5	17	1.11	0.95
5.35	5.5	6.2	1.0	2.5	11*	1.03	0.88
5.55	5.4	6.4	1.0	3.0	7.7*	1.06	0.90
5.75	4.5	6.3	1.9	3.0	6.8	1.08	0.75
5.95	3.95	6.2	2.2	4.0	4.8	1.03	0.66
6.0	2.75			5.5	3.8		
6.75	2.85	6.05	3.2	8.0	3.4	1.01	0.48
7.5	2.3			9.5	2.9		
8.6	2.8	5.9	1.5	9.0	2.4	0.98	0.47
10.55	2.75	5.6	1.4	8.5	2.8	0.93	0.46
11.7	2.45	5.9	0.8	7.0	2.5	0.99	0.41

* Calculated from the half-period.

studied the influence of the pH value of the medium on the course of chemical contraction and relaxation of collagen. For this purpose we soaked collagen fibres from the same rat for 30 min in a series of buffers according to Britton and Robinson in the pH range from 4.0 to 11.7 and examined them further in the usual way. The results are summarized in Table VI and show unambiguously the importance of the acidity of the solution used for pretreatment of the collagen fibre. Within the pH range from 7.5 to 11.7 the changes of the reactivity of the fibres are insignificant. In acid solutions (pH below 7.0), however, a gradually increasing influence upon the course of shrinkage and subsequent relaxation becomes manifest, and, according to the publications[3–4] quoted, this effect could be termed "rejuvenation" of the collagenous structure.

If we plot the rate constant k_{34} against the pH value of the buffer in which the fibres were previously incubated, we obtain a curve which in semilogarithmic coordinates shows two approximately linear parts. From Fig. 4 it is apparent that preliminary incubation of the fibres in buffers of pH 4–6 exerts a material influence on the rate of relaxation, whereas incubation in a medium of pH 7–11 is ineffective.

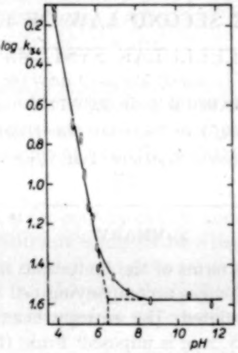

Fig. 4. Dependence of the rate constant k_{34} on the pH value of the Britton–Robinson buffer in semilogarithmic coordinates. In the figure are plotted the data of Table IV.

We conclude that the hydrogen ion concentration in buffers of pH 4–6 is sufficient for protonation of the basic groups in collagen and consequent structural changes, which manifest themselves, apart from other phenomena, also by a change in relaxability. It seems that the intersection of both linear parts of the graph of the dependence of log k_{34} on the pH value (Fig. 4), located at pH 6.5, corresponds to the isoelectric point of collagen, for which values most frequently given in the literature[13, 14] range from 6 to 7.5.

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SOME CONSEQUENCES OF THE APPLICATION OF THE SECOND LAW OF THERMODYNAMICS TO CELLULAR SYSTEMS

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(Received September 21st, 1959)

SUMMARY

Cellular division is examined in terms of the limitations imposed by the second law of thermodynamics. Entropy changes accompanying cell division are discussed and expressions for these are formulated. The entropy changes are summed and the limitation of the second law ($\Delta S > 0$) is imposed. From the resulting inequality the minimum amount of waste material accompanying cellular synthesis is computed. In addition certain conclusions are drawn as to the maximum rate of cell division.

INTRODUCTION

While the second law of thermodynamics as originally stated in terms of the impossibility of building certain kinds of machines has seemed very remote from biology, nevertheless, this law places very definite restrictions on the operation of living systems. The chief reason for the close relationship between the second law and living systems is the fact that when a cell ingests material from the environment and proceeds to grow into two cells there is a local decrease in entropy[1]. If no violation of the second law is going to occur, we must be able to make some statements restricting the process of cell growth. Recent attempts to apply information theory to living systems[2-4] as well as the intimate relation between information and entropy[5] suggest that the question of the relation between cell growth and the second law of thermodynamics be examined in some detail.

In order to study the problem, we will deal with the following idealized, but experimentally approachable situation. Consider a flask of volume V containing a dilute solution of small nutrient molecules. The flask is in thermal contact within an infinite isothermal reservoir at temperature T. The flask contains a single living cell of volume v. The process we wish to focus attention on is the growth and division of the cell into two similar cells. For convenience assume the further idealization that the cells are spherical during most of their life cycle. The process may be formally represented as

$$1 \text{ cell} + \text{nutrients} \longrightarrow 2 \text{ cells} + \text{waste products}$$

Since the initial cell acts as a catalyst for the overall process, and is effective unchanged at the end of the process, we may simplify the formalism and represent it as

$$\text{nutrients} \longrightarrow \text{cell} + \text{waste products}$$

For cells which grow in simple defined media the above formalism can be stated with some precision. For example for *Escherichia coli* grown in *C* minimal medium supplemented with glucose[6], we may write

$$\begin{aligned}&(3.22\cdot 10^9)\,C_6H_{12}O_6 + (1.94\cdot 10^9)\,NH_3 + (1.69\cdot 10^8)\,H_3PO_4 + (4.8\cdot 10^7)\,H_2SO_4 + \qquad (1)\\&(9.40\cdot 10^9)\,O_2 \longrightarrow (1)\ \textit{E. coli} + (9.55\cdot 10^9)\,CO_2 + (1.48\cdot 10^9)\,CH_3COOH +\\&(2.11\cdot 10^9\ \text{End Products} + (13.63\cdot 10^9)\,H_2O\end{aligned}$$

The requirement that we wish to apply to the aforementioned process is that the second law of thermodynamics holds, that is for the entire system including the reservoir the entropy change is greater than or equal to zero for the synthesis of a living cell. Stating the requirement formally, we may write

$$\Delta S \geqslant 0 \qquad (2)$$

To get a more detailed idea of the entropy change, consider that in the process outlined the following occurs: (a) Nutrient is ingested by the cell. (b) This nutrient is either incorporated into the cell or reacted and given off as waste products. (c) Heat is given off by the reactions and is transferred to the isothermal reservoir.

To calculate the entropy changes we will consider cell production to consist of two linked processes.

Process I. Nutrient is concentrated in the cell and converted to cell material.

Process II. Nutrient is converted to waste material. Since nutrient and waste material both end up dissolved in the medium surrounding the cell we need not calculate the entropy change in the concentration of this part of the nutrient and subsequent dilution of the waste material.

Since the two processes are linked, the linkage can be expressed by a parameter f, where f represents the number of grams of waste product produced per gram of cell produced. If the cell mass is then m, fm grams of material will be given off in the synthesis of a single cell. The entropy changes are as follows:

1. The entropy decrease involved in condensing the nutrient molecules in the cell. This may be approximated by $\Delta S_c = -mR/M \log Cc/Cm$, where M is the average molecular weight of the nutrients, Cc is the concentration of material in the cell (g/l) and Cm is the concentration of nutrient in the medium. This expression is derived by assuming that the entropy can be approximated by the entropy of an ideal gas, that $V \gg v$, the volume of the container is much larger than the volume of the cell, and that $Cc \gg Cm$, the material is much more concentrated in the cell than in the nutrient solution.

2. The entropy decrease involved in ordering the nutrient molecules into a cellular structure. At the moment we may represent this as ΔS_0. This is a quantity related to calculated values of the information content of a cell. We may use the relationship

$$\Delta S_0 = kI \ln 2 \qquad (3)$$

where k is Boltzmanns constant and I is the information increase of the cell over the unordered component clusters of atoms. For convenience in later expression we shall define a normalized information density as $i = I/mN$ where N is Avagadro's number. The entropy change is then

$$\Delta S_0 = -kmNi \ln 2 \qquad (4)$$

3. The entropy change (probably increase) in converting nutrients to waste

products. If this is ΔSw entropy units per gram then the total change in the growth of a cell will be $fm\Delta Sw$.

4. The entropy change $\Delta Q/T$ involved in the transfer of heat to or from the reservoir. This change is given by

$$\frac{\Delta Q}{T} = -\frac{(m\Delta Hc + fm\Delta Hw)}{T}$$

where ΔHc is the enthalpy per gram for cell synthesis and ΔHw is the enthalpy per gram of waste produced. Thus, summing all the entropy changes, we get

$$\Delta S = -\frac{mR}{M}\log\frac{Cc}{Cm} - mRi\ln 2 + fm\Delta Sw - \frac{(m\Delta Hc + fm\Delta Hw)}{T} \geqslant 0 \quad (5)$$

Rearranging terms we get

$$-\frac{RT}{M}\log\frac{Cc}{Cm} - RTi\ln 2 - f\Delta Fw - \Delta Hc \geqslant 0 \quad (6)$$

where ΔFw is the free energy change per gram of waste produced. Since the first two terms are negative and ΔHc may be in principle either positive or negative (we will later attempt an approximate calculation of this quantity) but is probably small, the second law demands that ΔFw is negative and that f has a certain minimum value given by

$$f \geqslant -\frac{1}{\Delta Fw}\left[\frac{RT}{M}\log\frac{Cc}{Cm} + RTi\ln 2 + \Delta Hc\right] \quad (7)$$

The quantity f measures the minimum price that must be payed in converting unorganized medium into a highly organized cell. Before attempting to compute minimum values of f we must have some idea of the value of ΔHc, the enthalpy of formation of a cell. We reconsider the chemical equation (1) which we have written for the formation of an *E. coli*. We then must split this into two equations, one describing the cellular synthesis and the other describing the metabolic formation of waste products.

The equation describing cellular synthesis is

$$(0.97\cdot 10^9)\,C_6H_{12}O_6 + (1.73\cdot 10^9)\,NH_3 + (0.196\cdot 10^9)\,H_3PO_4 + \quad (8)$$
$$(0.048\cdot 10^9)\,H_2SO_4 \longrightarrow 1\ \textit{E. coli} + 4.03\cdot 10^9\,H_2O$$

Including $4.03\cdot 10^9$ water molecules along with the synthesis of the cell is somewhat arbitrary; it is done to balance the chemical equations. (We shall later correct the enthalpy for this factor.) The result of this is to make the estimate of ΔHc too high. The atomic composition of the *E. coli* cell is $5.83\cdot 10^9$ carbon, $9.38\cdot 10^9$ hydrogen, $2.69\cdot 10^9$ oxygen, $1.73\cdot 10^9$ nitrogen, $0.169\cdot 10^9$ phosphorus and $0.048\cdot 10^9$ sulfur. If we know the types and distribution of bonds on both sides of the equation we can use the available data on heat of formation of bonds[7,8] to calculate the ΔHc of the process. From the macromolecular composition of the *E. coli* cell, its bond distribution has been computed[9,10]. The data are presented in Table I. The value of ΔHc as computed from these data is -682 cal/g. If we consider the formation of water as a linked reaction and use an approximate method to evaluate the ΔH of this reaction, the ΔHc is reduced to a value of -124 cal/g.

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[*] Bond distributions are calculated from the known information on the composition and the structural formulae of the constituents. Some relevant information is presented in ref. [9,10].

TABLE I

DISTRIBUTION OF COVALENT BONDS IN NUTRIENT MEDIUM AND IN *Escherichia coli*

Nutrients		*Cell and cellwater*	
C—H	$54.2 \cdot 10^8$	C—H	$71.03 \cdot 10^8$
C—C	$48.5 \cdot 10^8$	C—C	$37.94 \cdot 10^8$
C—N	0.0	C—N	$28.75 \cdot 10^8$
N—H	$51.9 \cdot 10^8$	N—H	$17.01 \cdot 10^8$
C=O	0.0	C=O	$13.37 \cdot 10^8$
C—O	$67.9 \cdot 10^8$	C—O	$12.25 \cdot 10^8$
P—O	$5.07 \cdot 10^8$	P—O	$5.14 \cdot 10^8$
O—H	$53.5 \cdot 10^8$	O—H	$5.79 \cdot 10^8$
C=C	0.0	C=C	$3.00 \cdot 10^8$
C=N	0.0	C=N	$3.00 \cdot 10^8$
P=O	$1.69 \cdot 10^8$	P=O	$1.71 \cdot 10^8$
C—S	0.0	C—S	$0.75 \cdot 10^8$
S—H	0.0	S—H	$0.17 \cdot 10^8$
S—S	0.0	S—S	$0.04 \cdot 10^8$
S=O	$0.96 \cdot 10^8$	S=O	0.0
S—O	$0.96 \cdot 10^8$	S—O	0.0
		Water	
		O—H	$80.6 \cdot 10^8$

We may now attempt to evaluate *f* minimum, by substituting observed and calculated values for the parameters in equation (7).

(a) *Cc* is the order of 250 g/l while *Cm* is the order of 5 g/l. (*Cm* is under the experimenters control and may be varied over a wide range. We shall later make use of this fact.) *M* may be taken as the order of 100 and we shall perform the calculation for $T = 300°$. The term $RT/M \log Cc/Cm$ is then 23.4 cal/g. (b) The term $RTi \ln 2$ is 159 cal/g based on the calculated information content of a 10^{-13} g cell as $4 \cdot 10^{10}$ bits[4]. (c) ΔHc has been calculated as — 124 cal/g. The quantity is very dependent on the nutrient medium the cells are grown in. In a very enriched medium ΔHc probably approaches zero. (d) The principle linked reaction in the case just discussed is the conversion of glucose to carbon dioxide and water. For a first approximation we may assume that ΔFw is the free energy of glucose oxidation. This free energy is — 691,000 cal/mole or 1860 cal/g.

Substituting in equation (7), we get

$$f \geqslant \frac{[58.4]}{1860} = 0.03 \tag{9}$$

Note that the quantity in the parenthesis is positive, indicating *f* is positive, that is that linked reactions must exist. While the calculated *f* value seems small, it is a minimum. The assumed value of ΔFw is high because the other linked reactions have a lower ΔF than the glucose oxidation. Experimentally for the example chosen *f* has a value 4.95, that is to say for each gram of cellular material synthesized 4.95 g of waste product (principally CO_2 and water) are given off. Thermodynamically *f* min is the order of 1/100th this value indicating of course that the process of *coli* duplication in minimal medium produces far more entropy than is required to satisfy the second law. From some published value on the heat given off by dividing bacterial cells[11] we previously calculated[4] that *coli* cells produced about 3.3 times the entropy required by a balance of the second law. Since the thermal measurements were made on cells growing in a complex medium, it is difficult to compare the two figures except to note that the cells seem to be vastly more efficient (as entropy sinks) when growing in a complex medium.

Equation (7) suggests an experimental check under certain extreme conditions. We may rewrite (7) as an equality:

$$f = \frac{-1}{\Delta Fw}\left[\frac{RT}{\mathrm{M}}\log\frac{Cc}{Cm} + RTi\ln 2 + \Delta Hc\right] + f' \tag{10}$$

where f' is a measure of the extra entropy produced or the inefficiency of the cell in ordering its constituents with the least possible change in the surroundings.

Now it seems clear that f' will not depend strongly on Cm, the concentration of nutrients, because any effects due to medium concentration should be covered in the term $RT/M \log Cc/Cm$. We have already pointed out that Cm is under the control of the experimenter. We may then measure f as a function of Cm and plotting f *versus* $\log Cc/Cm$ the slope will be $RT/\mathrm{M}\Delta Fw$ which gives an opportunity to measure the effective value of ΔFw. In cases where f' is the dominating term it will be difficult to determine the effect due to concentration but for cases of very enriched medium where f seems to be closer to f min the effect should be observable.

The necessary production of heat involved in the entropy balance imposes further restrictions on cell duplication. Specifically, since exothermic processes are a necessary part of cell duplication, the rate of duplication must be slow enough to dissipate the heat or else thermal inactivation of cell components will result. This may be seen by rewriting equation (5) as

$$\Delta Q \geqslant \frac{RT}{\mathrm{M}}\log\frac{Cc}{Cm} + RTi\ln 2 - fT\Delta Sw \tag{11}$$

where ΔQ equals $(\Delta Hc + f\Delta Hw)$. Now $m\Delta Q$ is the amount of heat produced in the dividing cell system in a single division time, I. If the average volume of the system during the division process is $3/2\ v$, the average rate of heat production per unit volume in the cell system, designated q, is $2/3\ m\Delta Q/v\tau$. If the average radius of the cell during the process is a, then assuming that the temperature steady state is reached rapidly in comparison to the change in size, we can use the theory of thermal conductivity to compute the temperature at the center of the sphere, Ti,

$$Ti = T + \frac{qa^2}{K} \tag{12}$$

where K is the thermal conductivity and T is the temperature of the environment. Substituting for q we get

$$\Delta T = Ti - T = \frac{-2\,ma^2}{3\tau vK}(\Delta Hc + f\Delta Hw) \tag{13}$$

In general if the cell is going to avoid disruptive effects due to heat $\Delta T < \beta$ where β is some temperature rise which empirically interferes with cell replication. Applying this to equation (13) and rearranging terms

$$\frac{\tau}{a^2} > -\frac{2\rho}{3\beta K}(\Delta Hc + f\Delta Hw) \tag{14}$$

where ϱ is the density of the cell m/v. Substituting reasonable values of the parameters*, we get

$$\tau > (12{,}400 + 181.200\,f)a^2$$

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* $K = 0.0014$ cal deg/cm sec; $\varrho = 1.05$ g/cm^3; $\beta = 5°$; $\Delta Hc = -124$ cal/g; $\Delta Hw = -1812$ cal/g.

Table II presents τ as a function of a for some extreme values of f. In principle Table II should hold for a tissue mass as well as a cell, provided that conduction is the only method available for getting rid of heat. It is then clear that as a cell or tissue gets larger than a millimeter or so in size it must develop methods of getting rid of heat or it must divide at a rather slow rate. This limitation stems only from the second law of thermodynamics and the auxiliary fact that living systems are subject to thermal inactivation.

TABLE II

MINIMUM DIVISION TIME OF CELLS AS A FUNCTION OF SIZE AND METABOLIC EFFICIENCY

a	For $f = 0.1$	For $f = 10$
1 μ	0.0003 sec	0.02 sec
10 μ	0.03 sec	2 sec
100 μ	3 sec	200 sec
1 mm	5 min	300 min
1 cm	5000 min	500 h
10 cm	833 h	2000 days

The main conclusion to be drawn at this point is that thermodynamic analysis can be useful in a detailed analysis on some living processes. At present experimental data is not oriented toward this type of treatment. The analysis itself is of course capable of much greater sophistication. Open fields for such investigation are irreversible thermodynamics[12] and the application of statistical mechanics to biological system[10].

ACKNOWLEDGEMENT

This work was supported by a Grant from the U.S. Public Health Service.

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Short Communications

The effect of inorganic ions on the crystallization of amylodextrin

There are three modifications of the crystalline structure of starch granules[1]. The A-type have been found in cereal starches, the B-type in tuber starches and the C-type, a mixed A- and B-type, in bean and root starches, though some exceptional cases have also been found[3-6].

As is well known, starchy materials usually give B-type crystals when they are retrograded at room temperature[7], although A-type crystals have been obtained under some conditions[7-11]. Therefore, it was considered that the crystalline type of native starch granules might be controlled by the conditions of crystallization of the starchy materials in the plant. In this communication, the effect of various inorganic ions on the crystallization of amylodextrin is reported.

The amylodextrin used in this experiment was obtained from a specific kind of sweet-potato starch syrup as reported in the previous paper[11]. The degree of polymerization of the amylodextrin was 12.6. It was a linear molecule and was hydrolyzed completely by crystalline sweet-potato β-amylase to maltose and a small amount of glucose. This material could be crystallized easily as the A-, B- and C-type under different conditions, but the degree of crystallinity was higher than that in native starch granules as judged by the intensities of the diffraction lines.

To 1.1 g of the amylodextrin (1.0 g on a dry basis) in a 5-ml conical flask, 1.5 ml water was added. The amylodextrin was dissolved by immersion of the flask in gently boiling water with shaking for 1.5 min. The clear solution thus obtained was cooled for several minutes in a bath of cold water kept at the crystallization temperature. The solution was still clear. An appropriate amount (0.3–4 mmoles, as a dry powder) of a salt was added to the solution. After the salt was dissolved, the flask was tightly stoppered and different flasks were placed in incubators at a constant temperature (7.5° ± 0.5°, 12° ± 0.5° and 22° ± 0.5°) for 5 days. The precipitated amylodextrin was collected by filtration on a suction filter and treated successively with 30 ml 60 % methanol and 50 ml 80 % methanol to remove the mother liquor and the added salt. The precipitate was dehydrated with absolute alcohol and washed with ether on the suction filter. The resulting amylodextrin powder was examined by an X-ray diffractometer (Shimadzu GX-II). X-rays were Cu K_{α} eliminating K_{β} with a nickel filter. The high tension voltage was 35 kV and the current 18 mA.

As shown in curve I of Fig. 1, a B-type amylodextrin was formed when crystallized from a pure aqueous solution under the conditions described above. On the other hand, C- or A-type amylodextrin was crystallized from NaCl. The X-ray patterns approached the A-type through the C-type with increasing concentrations of the salt, as shown in curves II–V of Fig. 1. The same phenomenon was also observed to different degrees in various other salt solutions as shown in Table I. The order of effectiveness of the salts in producing A-type amylodextrin crystals was found to be $Na^+ > Li^+, NH_4^+ > K^+ > Rb^+$ for the cation series (when tested as their chlorides) and $I^- > SCN^- > Br^- > Cl^- \gg SO_4^=$ in the anion series (when tested as their potassium salts). The orders of effectiveness was similar to the lyotropic series, though the order of Na^+ and Li^+, and of I^- and SCN^- were reversed in these series[12].

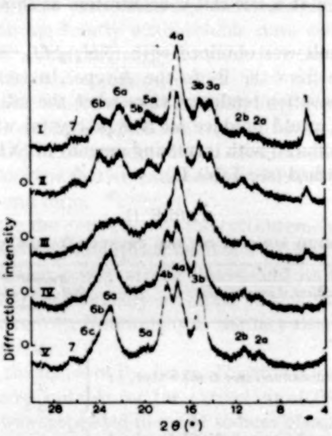

Fig. 1. X-ray-diffraction curves of amylodextrins crystallized from aq. NaCl. Concentrations (mmoles per 1.6 ml water containing 1 g amylodextrin) of NaCl were: I, none (B-type); II, 1 (C_b-type§); III, 1.5 (C_e-type§); IV, 2 (C_a-type§); V, 3 (A-type). The concentrations of amylodextrin were between 35.4 % and 38.5 % after the addition of the NaCl. This concentration change had no effect on the crystalline type, because from an aqueous solution of amylodextrin of below 46% concentration at 7.5°, the B-type crystal was always obtained.

TABLE I

CRYSTALLINE TYPES OF AMYLODEXTRIN CRYSTALLIZED FROM VARIOUS INORGANIC SALT SOLUTIONS

Crystallization temperature was 7.5°; in some cases 12°.

*Amount added salt (mmoles)**	*0*	*0.3*	*1*	*1.5*	*2*	*3*	*4*
	Crystalline type of amylodextrin						
NaCl	B		C_b	C_e	C_a	A	
NaCl (12°)	B		A				
LiCl	B		C_b		C_a	A	
NH_4Cl	B		C_b		C_a	A	
KCl	B		C_b	C_b	C_b	C_e	C_a
KCl (12°)	B	C_e	C_a				
RbCl	B				C_b	C_b	C_b
KI	B		C_e	C_a			
KCN	B		C_e	C_a			
KBr	B		C_e	C_e	C_a		
$\frac{1}{2}K_2SO_4$	B		B	B	B	B	
$\frac{1}{2}(NH_4)_2SO_4$	B		B		B	B	B

* mmoles dry salt added to the solution made by dissolving 1 g amylodextrin in 1.6 ml water.

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§ C-type was divided into three sub-types; C_a: near A-type, C_e: intermediate, and C_b: near B-type.

The crystalline types of freshly precipitated amylodextrin were not only influenced by salts as described above, but by the temperature of crystallization and the concentration of the amylodextrin[13]. When the crystallization was carried out at the higher temperature and at a higher amylodextrin concentration, the A-type amylodextrin was formed even at a low salt concentration as shown in the cases of NaCl and KCl in Table I.

An exceptional result was obtained with $(NH_4)_2SO_4$, which did not have any effect on the transition from the B- to the A-type. In fact, it rather showed the reverse effect for the transition tendency, since when the salt was added to a amylodextrin solution which would produce an A-type crystal without the salt, C- and B-type crystals were produced with increasing amount of $(NH_4)_2SO_4$, and the effects of other salts were abolished (see Table II).

TABLE II

THE EFFECT OF AMMONIUM SULFATE ON THE CRYSTALLIZATION OF AMYLODEXTRIN

Conditions of crystallization	*Amount* of $(NH_4)_2SO_4$ added (mmole)*	*Crystalline type*
1 g amylodextrin dissolved in 1.6 ml water, 22°	0	A
	1	C_a
	2	C_c
	3.5	B
1 g amylodextrin and 3 mmole NaCl dissolved in 1.6 ml of water, 7.5°	0	A
	3	B

* mmoles dry salt added to a solution made by dissolving 1 g amylodextrin in 1.6 ml water.

From these results, it seems that the various salts in living plant organs might affect the crystalline types of native starch granules.

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Received November 30th, 1959

Purification and some properties of a *b*-type cytochrome from *Sclerotinia libertiana*

Sofar, attempts to extract the *b*-type cytochromes other than cytochrome b_2[1] in a truly water-soluble state have been uniformly unsuccessful, although some of them have been obtained in an apparently water-soluble state with the aid of detergents such as sodium cholate[2–4]. This may suggest either that the *b*-type cytochromes are essentially water-insoluble haemoproteins, or that they may exist in the cell firmly bound to some hydrophobic substance such as lipid. Experiments reported here show that a *b*-type cytochrome can be obtained in a truly water-soluble state from a certain fungus which has been known to contain a lipase of extraordinary high activity. This finding seems to favour the view that at least a certain form of *b*-type cytochrome exist in the cells in a lipid-bound form.

The material used for the extraction of the cytochrome was a fungus *Sclerotinia libertiana* which shows a strong absorption band of a *b*-type cytochrome. This fungus has been known to produce a very strong lipase, and its lyophilized powder has recently become available commercially in Japan as a preparation of lipase (under the name of Lipase "Saiken"[*]). Noteworthy is the fact that this preparation shows almost no proteolytic activity.

The optimum pH for the lipase of the fungus lies at 6–8 (see ref. 5). This pH range was also found to be most suitable for the extraction of the cytochrome. The lyophilized fungal powder was suspended in 0.2 *M* sodium phosphate buffer, pH 7.0, and left overnight at room temperature (15°–20°). (A suitable ionic strength of the extracting medium was found to be 0.3 or higher.) The suspension was filtered through a Büchner funnel with the aid of Celite, and the resulting filtrate was dialysed against tap water overnight. The dialysed solution was passed through an aluminium oxide column which had been buffered with 0.2 *M* ammonium phosphate and washed with water. The cytochrome adsorbed on the column was washed with water and then eluted with 1.0 *M* ammonium phosphate buffer, pH 7.0. To the eluate was added solid $(NH_4)_2SO_4$ to 90 % saturation, the resulting precipitate was discarded by centrifugation, and the supernatant was dialysed against tap water for one day. After repeating

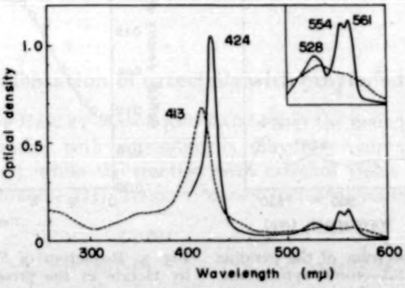

Fig. 1. Absorption spectra of *Sclerotinia* cytochrome. ——, reduced form; – – –, oxidized form. A more detailed absorption spectrum of the α- and β-absorption peaks is shown in the inserted graph.

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the procedure of adsorption and elution using the aluminium oxide column, the eluate containing the cytochrome was saturated with $(NH_4)_2SO_4$, the precipitate formed was removed by centrifugation, and the supernatant was dialysed against tap water. The cytochrome present in the solution was again adsorbed onto the aluminium oxide column and eluted with 10% saturated $(NH_4)_2SO_4$ (pH 5.6). The eluate obtained was light red in colour which turned into pink when treated with a small amount of $Na_2S_2O_4$. In Fig. 1 are reproduced the absorption spectra shown by the oxidized and reduced form of the cytochrome. While the oxidized form showed a sharp absorption peak at 413 mμ and two weaker bands at 532 mμ and 560 mμ, the $Na_2S_2O_4$-reduced form had sharp absorption peaks at 424 mμ, 528 mμ, 554 mμ and 561 mμ with a shoulder at around 534 mμ.

This feature of the absorption spectrum appeared to indicate that the solution we have obtained might have contained two cytochromes with the α-band lying at 561 mμ and 554 mμ, respectively. The solution was, therefore, subjected to a zone electrophoresis using a vertical starch column. Repeated experiments conducted under widely different conditions gave, however, no indication at all that the solution contained two different cytochromes. When the solution was shaken with a mixture of pyridine and 0.2 *N* NaOH (1:2, v/v), the water layer lost its colour, while the pyridine layer became red. When treated with $Na_2S_2O_4$, the pyridine layer showed an absorption spectrum having peaks at 419 mμ, 524 mμ and 556 mμ, which are characteristic for protohaemochromogen (Fig. 2). It may, therefore, be concluded that the cytochrome we have isolated was of the *b*-type, although its α-band differed from those of ordinary *b*-type cytochromes in showing two peaks even at room temperature (about 20°). That by our purification procedure the cytochrome had been obtained in a fairly pure form may be evidenced by the fact that the ratio of $E^{\text{reduced}}_{561\,m\mu}/E^{\text{oxidized}}_{280\,m\mu}$ was

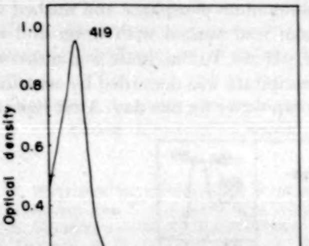

Fig. 2. Absorption spectrum of the pyridine haemochromogen of *Sclerotinia* cytochrome. The red colour of the cytochrome was easily extracted with alkaline pyridine. When $Na_2S_2O_4$ was added to the pyridine layer, the absorption spectrum of protohaemochromogen was seen.

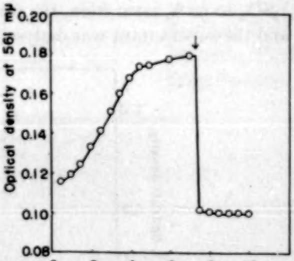

Fig. 3. Reduction of *Sclerotinia* cytochrome by lactate in the presence of baker's yeast lactic dehydrogenase under anaerobic conditions. When air was introduced as indicated by the arrow, the reduced cytochrome was rapidly oxidized. pH 6.5. Temp., 25°.

as high as 1.1 (see Fig. 1), which is comparable to the ratio of $E^{\mathrm{reduced}}_{550\,\mathrm{m}\mu}/E^{\mathrm{oxidized}}_{280\,\mathrm{m}\mu}$ obtained for a typical cytochrome *c*[*].

The *b*-type cytochrome was reduced by lactate in the presence of baker's yeast lactic dehydrogenase[6–9] under anaerobic conditions, but not under aerobic conditions. The enzymic reduction of the cytochrome was measured spectrophotometrically *in vacuo* in a Thunberg tube modified for spectrophotometry (Fig. 3). When air was introduced into the tube, the reduced cytochrome was immediately oxidized. The autoxidation of the cytochrome was not inhibited by 10^{-2} *M* KCN. Neither KCN nor CO caused any modification in the absorption bands as judged from the observation made with a microspectroscope. The biological function of the cytochrome, for which we propose the name "cytochrome *b*-561, 554 (*Sclerotinia libertiana*)"[10], is a matter for further investigation.

This investigation was supported in part by research grant (No. RG-5871) from the National Institutes of Health, U.S. Public Health Service.

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Received November 24th, 1959

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[*] It should be remarked that no *c*-type cytochrome has ever been extracted from this fungus.

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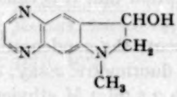
The condensation of catechols with ethylenediamine

It has been shown by Harley-Mason and Laird[1] that the main product of the reaction of ethylenediamine with adrenaline is dihydro-3-hydroxy-1-methylpyrrolo-(4,5-*g*)-quinoxaline (I), while the reaction with catechol yields 1,2,3,4-tetrahydro-1,4,5,8-tetraaza-anthracene (II). Harley-Mason (personal communication) further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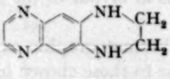
II

found that the main product formed from noradrenaline is identical with that formed from catechol and that the elimination of the β-ethanolamine side chain of noradrenaline occurring during this reaction depends on the presence of the β-hydroxyl group.

Several paper-chromatographic studies of the reaction products obtained from adrenaline and noradrenaline have been reported. They indicated the presence of a variety of compounds. Thus BURN AND FIELD[2] found two fluorescent derivatives of adrenaline and two of noradrenaline, while YOUNG AND FISCHER[3] described the formation of three derivatives from adrenaline and two from noradrenaline. According to YAGI AND NAGATSU[4] the condensation product of adrenaline separates into two spots of which the fainter one is identical with the main product of noradrenaline. NADEAU AND JOLY[5], finally, detected no less than ten different components derived from adrenaline and about the same number derived from noradrenaline.

In at least some of these experiments concentrations of catecholamines and ethylenediamine were employed which diverged greatly from those specified for the fluorimetric estimation[6] and which might therefore have been more favourable for the occurrence of side reactions. Moreover, the possibility cannot be excluded that the primary condensation product underwent decomposition either during the preliminary processing or during chromatography itself.

On the basis of the reaction mechanisms elucidated by HARLEY-MASON it is to be expected that the condensation product of 3,4-dihydroxymandelic acid with ethylenediamine would be identical with those formed from catechol and noradrenaline, but different from those of 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylalanine which do not possess the β-hydroxyl group in the side chain. This assumption is supported by the partition of the condensation products between an aqueous and an isobutanol phase: whereas the product from 3,4-dihydroxymandelic acid, like that from catechol or noradrenaline, is almost quantitatively extracted into isobutanol, those from 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxyphenylalanine remain largely in the aqueous layer[7].

The possible identity of the condensation products of catechol, noradrenaline and 3,4-dihydroxymandelic acid has now been further investigated by a comparison of their fluorescence spectra and by paper chromatography.

For the study of the fluorescence spectra 0.35 μg catechol or equivalent quantities of noradrenaline or 3,4-dihydroxymandelic acid, in 10 ml aqueous solution, were condensed with ethylenediamine under the conditions described by WEIL-MALHERBE AND BONE[6]. The resulting isobutanol extract was examined in the Aminco-Bowman Spectrophotofluorometer. The three extracts had identical maxima of activation and fluorescence at 420 and 485 mμ, respectively. The fluorescence curves rose sharply from zero at 450 mμ to the maximum and returned more gradually to zero at 590 mμ. After correction for slight differences of intensity the three curves were completely superimposable. In contrast to the slight asymmetry of these fluorescence curves, the fluorescence curve of the adrenaline derivative (peak at 525 mμ) was perfectly symmetrical, which is in favour of the assumption that it is caused by a single fluorescent product.

For the paper-chromatographic experiments the condensation was carried out under conditions close to those chosen for the fluorimetric assay: an aqueous solution containing 20 μg in 50 ml was incubated with 2.5 ml 2 M ethylenediamine dihydrochloride and 3.5 ml ethylenediamine at 55° for 30 min. After cooling the mixture was saturated with NaCl and extracted with 30 ml isobutanol. The isobutanol extract was evaporated *in vacuo* to dryness at 30° in a rotary "flash"-evaporator, the residue dissolved in small portions of methanol and concentrated in a test tube *in vacuo* to about 0.3 ml. In view of the reported photosensitivity of some of the condensation products[8], amber or red glassware was used throughout. The application of the solution (0.05 ml) to the paper (Whatman No. 3) was carried out in red light and the chromatography jars were kept under a darkened hood. Out of ten different solvent systems the best results were obtained with a mixture of isobutanol–ethylenediamine–water (50:3:20; organic phase) used in the descending direction. As shown in Table I

TABLE I

PAPER CHROMATOGRAPHY OF ETHYLENEDIAMINE CONDENSATION PRODUCTS

Solvent: isobutanol–ethylenediamine–water (50:3:20), descending technique.

Substance	*Number of spots*	R_F	*Fluorescence*
Adrenaline	1	0.30–0.34	Yellow
Noradrenaline	3	(1) 0.07	Green-yellow
		(2) 0.15–0.22	Light green
		(3) 0.55–0.59	Blue-green
Catechol	2	(1) 0.15–0.22	Light green
		(2) 0.55–0.59	Blue-green
Dihydroxymandelic acid	2	(1) 0.15–0.22	Light green
		(2) 0.55–0.59	Blue-green

the condensation product of adrenaline gave a single spot with intense yellow fluorescence. Each of the products formed from catechol, noradrenaline and 3,4-dihydroxymandelic acid yielded two indentical spots; a third faint spot with low R_F value was observed among the noradrenaline derivatives.

These results suggest that the condensation of ethylenediamine with adrenaline leads to a single fluorescent product and that side reactions are negligible. In the case of catechol, noradrenaline and 3,4-dihydroxymandelic acid two main products which appear to be identical for the three compounds are formed.

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Received September 16th, 1959

Biochim. Biophys. Acta, 40 (1960) 351–353

Hexose monophosphate pathway in thyroid tissue

Many of the substrates of the tricarboxylic acid and EMBDEN-MEYERHOF cycles are oxidized by appropriately supplemented thyroid homogenates. However, lactate was the only one of these substrates which was oxidized by thyroid tissue slices[1]. Although the existence of glucose-6-phosphate dehydrogenase is suggested by some experiments[2], no report has yet appeared on the presence of an hexose monophosphate pathway in the thyroid. In this study, the consumption of glucose by thyroid slices is demonstrated, and evidence is presented for the existence in this tissue of an important oxidative pentose phosphate cycle.

[1-^{14}C]glucose and [6-^{14}C]glucose (Nuclear-Chicago Corporation) were diluted with non-labeled glucose. The radioactivities of washed recrystallized glucosazones and $BaCO_3$, plated on copper planchettes, were determined with a windowless automatic gas-flow counter (Tracerlab). Duplicate samples corrected for background were adjusted to a 5-mg standard[3]. Sheep muscles and thyroids were sliced with a Stadie-Riggs slicer, washed with Krebs–Ringer Bicarbonate, and distributed at random in duplicate Erlenmeyer flasks. One flask contained [1-^{14}C]glucose and another [6-^{14}C]glucose. The two experiments were directly comparable. After thorough flushing with O_2–CO_2 (95:5) for 7 min, the flasks were incubated in a shaker at 37.5° with this atmosphere for 30, 60, or 120 min. $^{14}CO_2$ was counted as $BaCO_3$ for 1,000 counts[4]. The mean of 4 different samples was used as the specific activity of CO in each flask. Three similar double experiments were made with sheep neck-muscle slices, and another control with two hemidiaphragms of a rat.

$^{14}CO_2$ is evolved when labeled glucose is incubated in the presence of thyroid slices (Table I). The ratio of $^{14}CO_2$ evolved from [1-^{14}C]glucose to that from [6-^{14}C]-glucose was 19.5 after 30-min incubation. When the incubation was prolonged, more CO_2 was evolved, but the ratio C_1/C_6 decreased markedly[5,6]. There is thus a preferential cleavage of the C_1 over the C_6 of glucose, indicative of an active hexose monophosphate pathway. If glucose were metabolized entirely via the glycolytic and citric acid pathway, a ratio of I would have been observed, as in muscle[6,7]. If one

TABLE I

Each flask contained 10 ml of Krebs–Ringer bicarbonate buffer (pH = 7.4), 124.6 µmoles glucose and approximately 1.40 g of tissue slices (wet wt.). Specific activities in counts/min/5 mg glucosazone were: [1-^{14}C]glucose, 28,000; [6-^{14}C]glucose, 107,940.

Tissue	*Incubation time (min)*	*Relative specific activity formed from**		*Ratio C_1/C_6***	*Number of experiments*
		[1-^{14}C]glucose	*[6-^{14}C]glucose*		
Thyroid	30	0.482 ± 0.040	0.024 ± 0.006	19.5 ± 5.2	5
Thyroid	60	0.657 ± 0.043	0.062 ± 0.010	10.7 ± 2.2	6
Thyroid	120	0.972 ± 0.145	0.265 ± 0.055	3.78 ± 1.0	5
Sheep muscle	60	0.147	0.162	0.91	1
Sheep muscle	60	0.165	0.130	1.27	1
Sheep muscle	60	0.172	0.112	1.55	1
Rat diaphragm	60	1.26	1.20	1.05	1

* Relative specific activity, expressed in per cent equals:

$$\frac{\text{Specific activity of } CO_2 \text{ in counts/min/}\mu\text{mole/g tissue}}{\text{Specific activity of glucose in counts/min/}\mu\text{mole glucose}}$$

** Ratio C_1/C_6: Means and standard errors of the ratios found for each pair of experiments.

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makes the assumptions that there is no randomization of isotope from C_1 to C_4, C_5 or C_6, and that the oxidation of C_1 of glucose via the hexose monophosphate pathway predominates over that of carbon C_2 and C_3, we can calculate[8] that 75 % of the CO_2 produced from glucose would come via this pathway. However, these assumptions are doubtful[5,8] and this percentage is thus considered as semi-quantitative[9].

The existence of an active pentose pathway in the thyroid tissue implies a supply of reduced triphosphopyridine nucleotide, which is, in fact, required in the deiodination of iodotyrosines[2,3] by a microsomal enzyme and may be the most efficient coenzyme for iodide binding to protein in the thyroid homogenate[10].

Since the hexose monophosphate pathway seems to be an active metabolic route for glucose in the adrenal gland[7] and in testis[11], the possibility of this being a common property of endocrine glands should be considered.

The author wishes to express his appreciation to Dr. R. Spiro for his advice. The author is a recipient of a U.S. Public Health Service Fellowship.

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Received November 28th, 1959

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Biochim. Biophys. Acta, 40 (1960) 354–355

Intracellular ribonuclease from *Bacillus subtilis*

Nishimura and Nomura previously reported that *Bacillus subtilis* accumulated large amounts of ribonucleases in a culture medium; one of these enzymes was highly purified[1,2]. The specificity, molecular weight and immunological nature of these extracellular enzymes have also been described[3].

The present report deals with the presence of an intracellular RNase in this organism which has quite different properties from the extracellular RNases. A lysozyme lysate was prepared from bacteria which had been harvested from the culture medium (70 h) by centrifugation, and washed 3 times with 0.05 *M* phosphate buffer, pH 7.3. The lysate showed a small amount of RNase activity, measured by a modi-

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Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; EDTA, ethylenediaminetetracetic acid.

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fication of the method of **Frish-Niggemeyer and Reddi**[4]. This activity was not inhibited at all by the addition of anti-extracellular RNases which had been shown substantially to inhibit extracellular RNases.

The RNase in the lysate was precipitated at pH 4, and completely inactivated by heating at 100° for 5 min. As shown in Fig. 1, the pH optimum of the enzyme was about 5.8 whereas that of the extracellular RNases were 7.5. The intracellular RNase was completely inhibited by the addition of $1.7 \cdot 10^{-3}$ *M* EDTA, although the same concentration of EDTA had no effect on extracellular RNases as indicated in Table I.

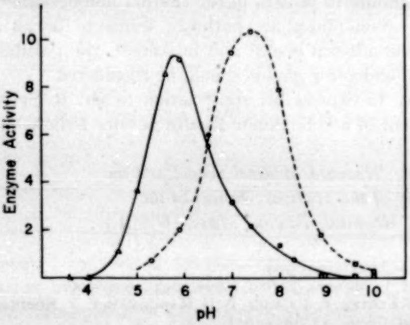

Fig. 1. pH-activity curves of intracellular and extracellular RNases. The final concentration of the various buffers was 0.067 *M*. △, Acetate; ○, phosphate; □, carbonate–bicarbonate; —, intracellular RNase; - - -, extracellular RNase (peak II).

TABLE I

EFFECT OF EDTA ON INTRACELLULAR AND EXTRACELLULAP RNASE

EDTA (M)	*Relative activity*		
	Extracellular RNase peak I	*Extracellular RNase peak II*	*Intracellular RNase*
$1.7 \cdot 10^{-2}$	—	100	0
$1.7 \cdot 10^{-3}$	100	100	0
$1.7 \cdot 10^{-4}$	100	100	58
0	100	100	100

Intracellular RNase was purified about 17 times by the following method: The lysate was centrifuged for 20 min at 27,000 × *g* and the supernatant fractionated with $(NH_4)_2SO_4$. The precipitate between 0.4 and 0.7 saturation at pH 5.6 was collected, dissolved in a small volume of water, and dialyzed overnight at 4°. 1 vol. 10 % streptomycin was added at pH 7.3 to 10 vol. of this dialysate. After standing for 2 h, the precipitate formed was removed by centrifugation. At this point the recovery of the enzyme in the supernatant was about 70 %. The enzyme was then precipitated at 0.7 saturation with $(NH_4)_2SO_4$. The precipitate was taken up in a small volume of water, dialyzed to remove salt and fractionated on the starch-electrophoresis apparatus (2 cm² × 40 cm). As shown in Fig. 2, the intracellular RNase could be separated from the phosphodiesterase which was in the original lysate.

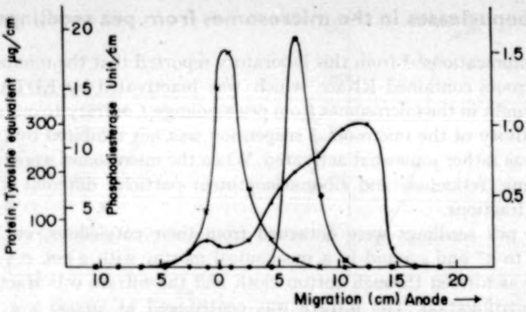

Fig. 2. Zone electrophoresis of intracellular RNase. The runs were made at 4° with a potential gradient of 7 V/cm for approximately 5.5 h. The starch was treated with 0.05 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.3. The charged enzyme preparation contained protein, 2,700 µg (tyrosine equivalent); RNase, 5.0 units (the activity corresponding to 1 µg of crystalline pancreatic RNase at optimum pH was defined as one RNase unit), and 85 phosphodiesterase units determined in a reaction mixture which contained 1 µmole Ca[bis(*p*-nitrophenyl)phosphate]$_2$, 50 µmoles tris(hydroxymethyl)aminomethane, pH 8.3, and aliquot of the enzyme in 2 ml of water. The amount of enzyme which caused an absorbancy increment at 440 mµ of 0.05 in 1 h at 30° was defined as one enzyme unit. ○, RNase; ×, phosphodiesterase; ●, protein.

Purified RNase produced all four constituent mononucleotides from yeast RNA. Nucleosides or free bases could not be detected. All four cyclic mononucleotides could be isolated from the dialysate, when yeast RNA was hydrolyzed by this enzyme in cellulose tubing suspended in a large volume of water. The identification of these cyclic mononucleotides was made by the method of MARKHAM AND SMITH[5].

The total activity of extracellular alkaline RNases in the culture medium after 70-h incubation was about 500 times larger than that of intracellular acid RNase in the cells. On the other hand alkaline RNases which were detected in the cell lysate were less than 1 % of the intracellular acid RNase, although the neutralizing units for anti-extracellular RNases was much larger than a theoretical value calculated from the enzyme activity. All of the intracellular acid RNase was contained in the supernatant fraction, and could not be detected in cell debris. These facts indicated the presence and distribution of two types of RNases, *i.e.* alkaline and acid RNases, in this organism which were different from the two RNases described for mammalian tissues[6,7].

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Received December 8th, 1959

Ribonucleases in the microsomes from pea seedlings

Previous communications[1,2] from this laboratory reported that the microsomes from young wheat roots contained RNase which was inactivated by EDTA. A similar enzyme was sought in the microsomes from pea seedlings. Contrary to our expectation the RNase activity of the microsomal suspension was not inhibited by the addition of EDTA; it was rather somewhat activated. When the microsomes were fractionated into endoplasmic reticulum and ribonucleoprotein particles different RNases were found in each fractions.

3-day-old pea seedlings were detached from their cotyledons, cut into small pieces, cooled to 5° and ground in a mechanical mortar with 2 vol. 0.5 *M* sucrose. The mixture was filtered through cotton cloth and the filtrate was fractionated by differential centrifugation. The filtrate was centrifuged at 16,000 × *g* for 15 min and the mitochondrial sediment removed. The supernatant was centrifuged at 50,000 × *g* for 30 min in a Spinco preparative centrifuge (with No. 30 rotor) to give a sediment of microsomes (endoplasmic reticulum and a part of ribonucleoprotein particles). The supernatant was further centrifuged at 100,000 × *g* for 120 min to give a sediment of ribonucleoprotein particles.

RNase in the endoplasmic reticulum

Washed microsomes were suspended in water and 0.2 vol. butanol was added to the suspension. After sonic oscillation for 10 min, NaCl was added to 0.15 *M* and the sonic oscillation repeated for 10 min. The suspension was centrifuged at 5,000 × *g* for 20 min, and the clear water phase separated from the butanol phase.

Some of the particles precipitated and others adsorbed on the butanol layer. The clear water phase was removed and dialyzed against water for 24 h in a cold room. The RNase in this solution was inactivated in 0.1 *M* acetate buffer, pH 6.0, with the addition of $2.5 \cdot 10^{-3}$ *M* EDTA, suggesting that this RNase requires a metal ion for its activity. For this reason, EDTA or deoxycholate which are generally used for the solubilization of the enzymes in the microsomes cannot be used for detecting this enzyme. The pH optimum was at 6.0 (see Fig. 1).

RNase in the ribonucleoprotein particles

The RNase in the ribonucleoprotein particles could be easily solubilized by metal-binding compounds, such as EDTA, deoxycholate, citrate or phosphate (see Table I). However, since the enzyme could not be completely solubilized by acetate buffer, it is clear that the enzyme was bound to particles. Apparently it does not require metal ions for its activity since it was not inhibited by EDTA. The enzyme solution which was solubilized from the ribonucleoprotein particles by citrate–phosphate, pH 6.0, was dialyzed against water for 24 h in a cold room. The pH optimum of this RNase was 5.6 (see Fig. 1).

From these results, it appears that the endoplasmic reticulum and ribonucleoprotein particles of the microsomes from pea seedlings contain different RNases which differ in pH optimum and in the effect of EDTA.

The RNase in the endoplasmic reticulum corresponds to the RNase which was previously found in the microsomes from young wheat roots[2].

Abbreviations: RNase, ribonuclease; EDTA, ethylenediaminetetraacetic acid.

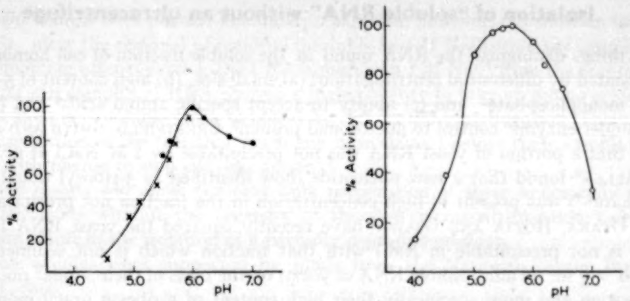

Fig. 1. pH-activity curve of endoplasmic reticulum RNase; ×—×, in 0.1 *M* acetate buffer; ●—●, in 0.1 *M* cacodylate buffer. ○—○, pH-activity curve of ribonucleoprotein particle RNase in 0.1 *M* citrate-phosphate buffer.

TABLE I

SOLUBILIZATION OF THE RIBONUCLEASE FROM THE RIBONUCLEOPROTEIN PARTICLES

Ribonucleoprotein particles from the pea seedlings were suspended in the indicated solutions. The suspensions were centrifuged at 10,000 × *g* for 30 min in order to sediment the particles. Both suspensions and supernatants were assayed for RNase activity. The RNase activity was determined spectrophotometrically in acetate buffer, pH 6.0. Activity is reported as absorbancy at 260 mμ.

Treatment	*Ribonuclease activity*		*Solubilization*
	Suspension	*Supernatant*	*%*
Acetate buffer, 0.1 *M*, pH 6	1.328 (100)	0.133	10
EDTA, 0.1 *M*; acetate buffer, 0.1 *M*, pH 6	1.472 (111)	1.480	100
EDTA, 0.01 *M*; acetate buffer, 0.1 *M*, pH 6	1.470 (111)	0.701	48
EDTA, 0.001 *M*; acetate buffer, 0.1 *M*, pH 6	1.338 (101)	0.271	20
Citrate-phosphate buffer, 0.1 *M*, pH 6	1.461 (110)	1.416	97
Phosphate buffer, 0.1 *M*, pH 6	1.272 (96)	1.220	96
0.2 % Deoxycholate	1.073 (81)	0.652	61

The RNase in the ribonucleoprotein particles may correspond to the RNases which were found in rat-liver microsomes by TASHIRO[3], in the mouse-pancreas microsomes by DICKMAN[4] and in the ribonucleoprotein of *Escherichia coli* by ELSON[5]. But these RNases were all latent and did not reveal their activity until the microsomes or ribonucleoprotein were treated with EDTA or urea. In the plant, on the other hand, the RNase in the ribonucleoprotein particles could also be solubilized by the addition of EDTA, but the suspension of the washed ribonucleoprotein particles themselves contained more of the RNase activity. Therefore, it is concluded that the RNase is bound on the surface of the particles.

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Received December 10th, 1959

Isolation of "soluble RNA" without an ultracentrifuge

Three things distinguish the RNA found in the soluble fraction of cell homogenates fractionated by differential centrifugation: (a) small size, (b) high content of 5-ribosyl uracil monophosphate[1], and (c) ability to accept specific amino acids[2] and transfer them under enzymic control to microsomal protein[3]. Crestfield, Smith and Allen[4] found that a portion of yeast RNA was not precipitable by 1 *M* NaCl at 0°. Davis and Allen[5] found that a new nucleotide (now identified as 5-ribosyl uracil monophosphate[6,7]) was present in high concentration in the fraction not precipitated by NaCl. Otaka, Hotta and Osawa[8] have recently equated the yeast RNA fraction which is not precipitable in NaCl with that fraction which is not sedimented at 105,000 × *g* for 90 min (soluble RNA of yeast) on the basis of their similar nucleotide composition and more specifically their high content of 5-ribosyl uracil monophosphate. Dunn[1] has shown that in mammalian cells soluble RNA is distinguished from microsomal RNA by its high content of 5-ribosyl uracil monophosphate. The present paper reports that the soluble RNA of mammalian cells as prepared by differential centrifugation is very similar or identical with the RNA which remains soluble in 1 *M* NaCl, as judged by its ability to accept amino acids under enzymic control.

Activating enzymes (pH 5 enzymes) were prepared from calf thymus and freed of RNA by treatment with protamine. After the respective cell fractions were separated by differential centrifugation, the RNA was isolated by extraction with phenol[9]. Part of the RNA was then further treated with cold 1 *M* NaCl[4]. The assay for the incorporation of amino acids into RNA was performed[10] using $[^{14}C]$algal-protein hydrolysate as the amino acid source.

TABLE I

INCORPORATION OF AMINO ACIDS BY VARIOUS RNA FRACTIONS FROM CALF THYMUS

RNA sample	*Specific activity**	
	No treatment	*RNA soluble after 1 M NaCl treatment*
Soluble RNA (small-scale preparation)	393**	—
Soluble RNA (large-scale preparation)	175	249
Non-nuclear RNA	134	578
Whole-cell RNA	88	430

* Counts/min/unit of absorbancy at 260 mμ.
** Average of duplicate assays.

Two samples of soluble RNA were studied. The large scale preparation was distinguished from the small scale preparation in that the 105,000 × *g* supernatant from the former preparation stood for several hours longer at 4° before extraction with phenol. The non-nuclear RNA was isolated from the cell fraction remaining after centrifugation at 3,500 × *g* for 10 min.

It is felt that the lowered ability of the bulk preparation of soluble RNA to take up amino acids as compared with the small-scale preparation (Table I) is probably due to enzymic degradation of the former sample because of a necessary delay in

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Abbreviation: RNA, ribonucleic acid.

mixing the sample with phenol. The bulk preparation, however, did show an increase in activity after the removal of material insoluble in 1 *M* NaCl from the sample. The RNA remaining after removing the RNA insoluble in 1 *M* NaCl from the non-nuclear and whole-cell samples of RNA showed a higher ability to take up amino acids than our best sample of soluble RNA obtained by differential centrifugation. This is probably a reflection of the fact that much less time elapsed before these samples were treated with phenol to stop enzymic action.

These results will allow the easy bulk preparation of these biologically active ribonucleic acids without the necessity of the slow ultracentrifugation technique which can result in the isolation of a partially degraded product.

These studies were supported by funds from the National Cancer Institute (Grant C-2896).

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Received December 3rd, 1959

Biochim. Biophys. Acta, 40 (1960) 360–361

The isolation of androsterone and aetiocholanolone from the urine of oophorectomised-adrenalectomised women

There have been several reports that patients with metastatic mammary cancer may continue to excrete various steroid hormones or their metabolites in the urine after the removal of the ovaries and the adrenal glands (for reviews, see ref. 1–3). The amounts of steroids found in the urine of such patients are very small and evidence for the identity of the compounds in question has generally been based on their chromatographic properties or, in the case of the oestrogens, on their biological activity[4].

Rigorous proof is needed for the identity of androsterone and aetiocholanolone since Δ 9–(11) artefacts produced by the dehydration of cortisone metabolites are very difficult to separate from androsterone and aetiocholanolone unless chemical modification is carried out[5]. Plantin *et al.*[6] found only Δ 9–(11) aetiocholenolone in urine obtained from adrenalectomized patients, a result which did not agree with the findings of Kellie and Wade[7], Bulbrook, Greenwood and Thomas[8] and Hobkirk[9,10] that such patients continue to excrete androsterone and aetiocholanolone.

An attempt was therefore made to isolate and identify the compounds in the androsterone and aetiocholanolone fractions prepared from urine from oophorectomized-adrenalectomized patients.

A pool of urine (70 l) was collected from women with metastatic breast cancer who had been subjected to oophorectomy and adrenalectomy at least one month previously. The urine was stored at —20° and thawed immediately before extraction. The methods used for the isolation of androsterone (3α-hydroxy-5α-androstan-17-one) and aetiocholanolone (3α-hydroxy-5β-androstan-17-one) were based as closely as possible on methods used for the detection and estimation of these compounds in urine. Conjugates were extracted and hydrolysed by the method of KELLIE AND WADE[7] and separated into a non-ketonic and a ketonic fraction by the method of MASON[11]. The total amount of Zimmermann-reacting material in the ketonic fractions was 37.4 mg, calculated in terms of dehydroepiandrosterone. After separating this fraction into 11-oxy and 11-deoxy-17-oxosteroids, using the ligroin–propylene glycol system of SAVARD[12], 3.2 mg of the latter were obtained. The compounds in this fraction were further resolved by running 500-μg portions (in strips across the paper) on a variant of the BUSH A paper chromatographic system[13], using isooctane–80 % aq. methanol. Compounds with the same mobility as standard androsterone and aetiocholanolone were eluted separately with ethanol. The pooled androsterone eluates were finally purified by gradient-elution chromatography on alumina[7]. Most of the elution solvents (ethanol; benzene) were removed at room temperature under a steam of nitrogen. The last traces of solvent were allowed to evaporate slowly *in vacuo*. A yield of 500 μg of white crystalline material was obtained. The aetiocholanolone fraction was treated in the same way, giving a final yield of 1200 μg of white crystals. Identification was by infrared spectrometry which was kindly carried out by Dr. T. F. GALLAGHER of the Sloan-Kettering Institute for Cancer Research, New York City. The spectra were identical with those of authentic androsterone and aetiocholanolone and there was no indication of the presence of unsaturated analogs.

The isolation of these compounds in a pure form in no way alters the previous conclusions of BULBROOK, GREENWOOD AND THOMAS[8] concerning the quantitative estimation of these compounds. Neither gradient elution from alumina nor paper chromatography on the BUSH A system is sufficient, used alone, for the separation of pure androsterone or aetiocholanolone in low-titre urine. If column chromatography alone is used, small amounts of other Zimmermann-reacting substances may be found in the androsterone eluate, and less frequently, in the aetiocholanolone eluate. Similarly, if paper chromatography is used alone, a compound with the same R_F as androsterone, but which is separable from it on alumina, may be found. In both cases, an over-estimate of the amounts of androsterone or aetiocholanolone is obtained.

There has been considerable controversy concerning the continued excretion of steroid hormones (or their metabolites) by women with cancer of the breast subjected to oophorectomy and adrenalectomy[3]. KELLIE AND WADE[7] found androsterone and aetiocholanolone in urine from such patients. BULBROOK, GREENWOOD AND THOMAS[8] found that most adrenalectomised patients continued to excrete aetiocholanolone but androsterone was rarely found. In all these cases, evidence for the identity of androsterone and aetiocholanolone was based on their behaviour on alumina columns and on paper-chromatographic systems. HOBKIRK[9,10] found androsterone in urine from 1 of 7, and aetiocholanolone in urine from 6 of 7 adrenalectomised women. Further characterisation of androsterone was carried out using a variety of paper-chromatographic systems and by examination of the absorption curve of the compound in H_2SO_4. The substance extracted from urine appeared to be authentic androsterone and no evidence was found for the Δ 9–(11) artefact. However, Plantin *et al.*[6] found only Δ 9–(11) aetiocholenolone in urine obtained from adrenalectomised women but it appears probable that this compound arose as a dehydration artefact, caused by the method of hydrolysis used for for the steroid conjugates by these workers. The isolation and characterisation of androsterone and aetiocholanolone by methods similar to those used for the determination of these compounds confirms the previous reports on their continued excretion by women subjected to oophorectomy and adrenalectomy. Furthermore, Gallagher[14] and his colleagues have also isolated these two steroids from similar sources.

The mean 24-h urine volume for the adrenalectomised patients was 1600 ml. From this figure it can be calculated that the mean daily excretion of androsterone was approximately 10 μg and of aetiocholanolone, 30 μg. These levels agree well with those found by Hobkirk[9].

The source of these compounds is still obscure. There is little likelihood that the 11-deoxy-17-oxosteroids arise from the maintenance doses of cortisone since Bradlow and Gallagher[15] have found that 11-oxysteroid hormones appear in the urine as compounds in which the 11-oxygen atom is retained, regardless of other changes in the molecule. The only report of the removal of the 11-oxygen atom is that of Miller and Alexrod[16], who make the surprising claim that cirrhotic rat liver converts cortisone to 6β, 21-dihydroxy-Δ^4-pregnene-3,20-dione (among other metabolites). However, since oestrogens and pregnanediol have also been found in urine from adrenalectomized patients the most likely source for so many steroids is adrenal tissue either left at operation or in the form of adrenal rests[3].

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Received December 21st, 1959

Protein determination on the basis of copper-binding capacity

Current photometric methods for the determination of protein rely either on the special properties of aromatic residues[1–3] or solely on the presence of polypeptide structures[4–6]. Those procedures dependent on aromatic groups are sensitive but vary in the absolute values given by different proteins. The procedures dependent on the complexing of copper by polypeptides, the so-called biuret methods[4,5], are more reliable but much less sensitive. NEILSEN's micro procedure[6] based on the biuret reaction has not been found generally useful in this laboratory because of turbidity, caused by precipitation of the water-insoluble copper diethyldithiocarbamate[7], and highly colored blanks, apparently caused by the formation of soluble copper-phosphate complexes[8]. This communication reports a rapid new procedure utilizing the biuret reaction, which has the reliability of the biuret method and a useful range of 0.05–1.0 mg protein.

Reagents

(a) Alkaline copper tartrate reagent: 10 ml 0.20 M $CuSO_4$ are added to 590 ml 0.167 N NaOH containing 2.0 g potassium sodium tartrate tetrahydrate. (b) Dowex-1 suspension: Bio-Rad AG1-X8, 200–400 mesh, chloride form*, is suspended in deionized water at a density of 200 mg/ml. (c) Color reagent: Sodium diethyldithiocarbamate is dissolved at a concentration of 1.0 mg/ml in a 0.025 % solution of crystalline bovine serum albumin. All of the reagents are stable for many weeks at room temperature.

Procedure

The protein solution to be analyzed is freed of salts and free amino acids by dialysis or the protein is precipitated with 0.5 vol. of 3 M trichloracetic acid. The concentration of salts and amino acids in the sample to be precipitated must be less than 0.4 M. Above this level, interference is most pronounced at the lower protein concentrations. Agreement between assay values obtained with aliquots of different sizes assures the absence of interference. The precipitated protein is sedimented by centrifugation for 5 min at 20,000 × g and then resuspended in 0.50 ml of deionized water. The protein suspension or 0.50 ml of dialyzed protein solution, containing 0.05–1.0 mg protein, is mixed in a centrifuge tube with 1.00 ml of alkaline copper tartrate solution, in which the protein precipitates readily dissolve. After at least 5 min at room temperature, 1.0 ml of Dowex-1 suspension is added, followed by 5.0 ml of color reagent. After mixing, the tube is centrifuged briefly at low speed and the supernatant solution is decanted into a cuvette. Absorbancies are determined at 446 or 486 mμ, depending on the amount of protein present, against a blank containing 2.5 ml of deionized water and 5.0 ml of color reagent. Any resin carried over in decanting settles rapidly and causes no interference with the photometry. The color is stable indefinitely.

Standard curves for the method are given in Figs. 1 and 2. The absorbancy response is linear up to 0.4 mg protein. At 446 mμ, which is the absorption maximum,

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* California Corporation for Biochemical Research. No cycling or other preparation of the resin is necessary. Equilibration with alkaline tartrate prior to use diminishes the effects of interfering substances but also reduces the sensitivity of the method.

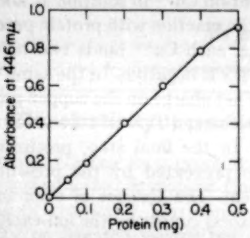

Fig. 1. Standard curve for the procedure at 446 mμ. The points shown are the means of values obtained for the five crystalline proteins of Table I. The instrument used was a Coleman Junior Spectrophotometer.

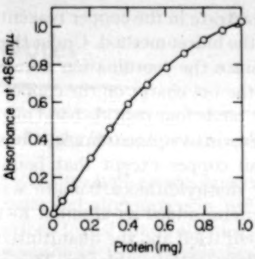

Fig. 2. Standard curve for the procedure at 486 mμ. Other conditions as in Fig. 1.

this corresponds to the most useful range of the photometer. The sensitivity of the method may be increased somewhat by using a smaller volume of a more concentrated color reagent and a suitable photometer.

The reliability of the method with different proteins is indicated by the data given in Table I. As was expected, the new procedure and the biuret method have similar reliabilities, while the sensitivity of the new procedure approaches that of the LOWRY method.

TABLE I

COMPARISON OF FOUR ANALYTICAL PROCEDURES IN THE DETERMINATION OF FIVE CRYSTALLINE PROTEINS

The crystalline proteins used were purchased from Nutritional Biochemicals Corporation. Dry weights were determined by drying samples to constant weight at 30° over Al_2O_3. The absorbancy values given are based on data sufficient to insure random errors smaller than 2 %.

Protein	*Apparent protein content of solutions containing 1.00 mg/ml**			
	*Estimation based on absorbancy at 280 mμ***	LOWRY *method**** (*ref. 3*)	*Biuret method**** (*ref. 5*)	*Present method****
Edestin	0.69	0.91	1.04	1.00
β-lactoglobulin	0.73	0.67	0.93	0.93
Bovine serum albumin	0.52	0.97	0.95	0.97
Pepsin	1.09	1.18	0.94	1.04
Lysozyme	1.97	1.22	1.14	1.05
Mean deviation (as % of mean)	42 %	17 %	7 %	4 %
Sensitivity (absorbance units/mg in the final volumes stated)	0.43	2.6	0.05	2.0

* The mean absorbancy value per mg for each method was taken as the standard for that method.

** Determined using the Beckman Model DU Ultraviolet Spectrophotometer, final vol. 3.0 ml.

*** Determined using the Coleman Junior Spectrophotometer, final vol. 7.5 ml. Absorbancy values were determined at the wavelength of the absorption maximum for each method.

The tartrate in the copper reagent serves to maintain Cu^{++} in solution at alkaline pH, as in the biuret method. Under these conditions the reaction with protein proceeds rapidly. Since the coordination number of Cu^{++} is 4, each Cu^{++} binds two tartrate ions, and the net charge on the copper-tartrate complex is negative. In the same way, each Cu^{++} binds four peptide bond nitrogens, but the net charge on the copper-protein complex is positive. Accordingly, the strong anion exchanger Dowex-1 removes from solution all copper except that bound by protein. In the final step, precipitation of copper diethyldithiocarbamate was found to be prevented by the presence of excess protein, added as albumin in the color reagent. The absence of color in the color reagent itself and the quantitative removal of excess copper by the ion-exchange resin result in colorless blanks. This circumstance assures high sensitivity for small amounts of protein and minimizes errors caused by inaccuracies in procedure.

In summary, a quantitative analytical procedure based on the copper-binding capacity of protein has been developed. Protein-complexed copper is determined colorimetrically with diethyldithiocarbamate after removal of excess copper by an ion-exchange resin. The procedure has 40 times the sensitivity of the biuret method and equal reliability.

This investigation was aided by grants from the National Science Foundation and the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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Received December 12th, 1959

Preliminary Notes

The elasticity-increasing property of elastomucoproteinase

Pancreatic elastase is a proteolytic enzyme which dissolves elastin specifically[1–3]. Other proteolytic enzymes of the pancreas, trypsin and chymotrypsin, are without elastolytic activity[4, 5]. Elastomucoproteinase described by us[1] shows also no elastolytic activity on isolated elastin but dissolves a mucoprotein from bovine ligamentum nuchae and from human aorta which on the basis of the Molisch-Szára reaction differs from the mucoprotein dissolved from native collagen.

We succeeded in separating elastomucoproteinase from collagen mucoproteinase preparatively as well as through paper electrophoresis. The elastolytic activity of elastomucoproteinase on different substrates was found to be negligible compared with elastase (Table I).

TABLE I

ELASTOLYTIC ACTIVITY OF ELASTOMUCOPROTEINASE AND ELASTASE

Substrate	*E.U./mg**	
	elastomuco-proteinase	*elastase*
Bovine ligamentum nuchae (powdered)	4	80
Elastin from ligamentum nuchae**	4	130
Elastin from bovine aorta**	4	130

* Elastolytic units[2].

** Elastins were prepared by the method of PARTRIDGE AND DAVIS[3]

Since elastomucoproteinase does not digest either elastin or collagen but dissolves a mucoid, one can suggest that this dissolved mucoid may stabilize the elastic fibres.

The effect of elastomucoproteinase was therefore investigated upon the tensile properties of ligamentum nuchae, of human aorta and carotid artery. Load–extension curves were measured before and after treatment by the enzyme.

Strips were cut from ligamentum nuchae parallel and across the carotid artery corresponding to the direction of the fibres and the work necessary to stretch the material to 30 % extension was determined[6] in Ringer solution. Enzyme treatment was carried out for 3 h at 37° at pH 7.4, in 0.025 *M* veronal–acetate buffer, at an enzyme concentration of 0.1 mg/ml. Suitable control experiments were carried out without elastomucoproteinase and with crystalline trypsin. The effect of enzyme is given by WOOD's[6] parameter $R = (W_2 - W_1)/W_1$ in which W_1, W_2 represent the work required for 30 % extension before and after enzyme treatment. Hysteresis was not a factor, since the load-extension curve was also measured before enzyme treatment.

The value of *R* using ligamentum nuchae was found to be constant, —0.20, if the wet weight of the strips did not exceed 25–35 mg. The *R* value is negative which denotes that the strip has been weakened by the treatment, *i.e.* the stability of the fiber decreased. In experiments with human carotid artery the age of the subject and the pathologic conditions influenced a great deal the effect of the elastomucoproteinase. In carotid arteries from human cadavers in which the internal layer was covered with atheromas the strip became more extensible, *i.e.* more elastic, after enzyme treatment as compared with the carotid from healthy and young individuals, *i.e.* the negative value of R increased in individuals with atherosclerosis. Investigations were performed with 36 human carotid arteries and in Table II values of R belonging to different age groups are exemplified.

TABLE II

EFFECT OF ELASTOMUCOPROTEINASE ON HUMAN CAROTID ARTERIES

Age of subject (years)	*Condition*	R	
		Control: buffer only	*elastomucoproteinase*
19	normal	0.0	— 0.20
30	normal	0.0	— 0.21
44	slight alteration	0.0	— 0.35
70	atherosclerosis	0.0	— 0.45
73	atherosclerosis	0.0	— 0.50
93	atherosclerosis	0.0	— 0.55

Several workers[7–9] have been engaged in the study of the changes of elastic extensibility in relation to atherosclerosis. It was generally observed that with age and with arterio- or atherosclerosis the elastic extensibility of blood vessels was greatly decreased. Our experiments confirm this observation. However, following the effect of elastomucoproteinase the decreased extensibility of the aged arteries improved and their elastic extensibility was regained.

Trypsin had no effect whatever on the tensile properties of blood vessels.

The mucoprotein dissolved by elastomucoproteinase[2] contains hexuronic acid, hexosamine and neuraminic acid and differs from the mucoid$_2$ isolated from bovine Achilles tendon with the aid of collagen-mucoproteinase[10]. The mucoid$_2$ was shown to be a stabilizing factor of collagen fibres[11] and consisted mainly of neutral heteropolysaccharides.

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Received March 9th, 1960

On the structure of lysopine, a new amino acid isolated from crown gall tissue[*]

One of us (C.L.) has recently reported that crown-gall tissue of salsify (*Scorzonera hispanica*), tobacco (*Nicotiana tabacum*), Virginia creeper (*Parthenocissus tricuspidata*) and Jerusalem artichoke (*Helianthus tuberosus*), cultivated *in vitro* contains large quantities of an "unknown" amino acid, not found in normal tissue and present only in the free state[1]. This substance was obtained in crystalline form [m.p. 157–160°, followed by decomposition at 240°); $[\alpha]_D + 18 \pm 1°$ in water (*c*, 1.40)] after purification on Permutite C 50 and by preparative paper chromatography; with ninhydrin, this compound gives a violet color and has the following R_F values: 0.09 in butanol–acetic acid–water (4:1:5) and 0.75 in phenol/NH_3[1,2].

On a Moore and Stein column of Amberlite CG-120[3], it is eluted like alanine, between glycine and valine.

A preliminary investigation of this new amino acid has shown that it was neutral, that it was not an α-amino acid, and that half of its nitrogen was present as an $-NH_2$ group[2].

A more detailed chemical examination revealed the probable presence of two carboxyl groups, one $-NH_2$ group, and one CH_3–C grouping and absence of OCH_3 and N–CH_3.

The determination of the exact molecular formula was difficult because of the presence of water or alcohol of crystallisation which was very tenaciously retained and because neither on acetylation nor on methylation homogenous crystalline preparations were obtained. Nevertheless, the molecular formula $C_9H_{18}N_2O_4$ was considered the most probable.

An examination of the ethyl ester of the amino acid by mass spectrometry with a CEC 21–103 C mass spectrometer equipped with a heated inlet system operated at 140° gave the following information:

The spectrum of the ester exhibited intense peaks at m/e 84, 156, 184, 201 and a small peak at 274, accompanied by an even smaller one at 275, the intensity of which, relative to the others, showed the changes, on variation of the ionizing conditions, characteristic[4] for $M + 1$ peaks. The molecular weight of the ester is therefore 274. This is confirmed by the strong peak at 201, which corresponds to the loss of the carbethoxy group, one of the most important fragmentation processes for ethyl esters of α-amino acids. This fragment decomposes further to mass 184, confirmed by the presence of a metastable peak[5] at 169 (calcd. 168.4), corresponding to the loss of NH_3 which indicated the presence of a primary amino group. The fragment 184 decomposes further to mass 156 (loss of 28 mass units), suggesting the presence of a second carbethoxy group ($-COOC_2H_5 \rightarrow -COOH + C_2H_4$). The corresponding metastable peak for this process is found at 133 (calcd. 132.3).

The most intense peak of the spectrum is found at mass 84 ($C_5H_{10}N$) indicating that the two nitrogen atoms are originally attached to a common C_5-segment, probably like in lysine, where the strongest peak also is mass 84 (ref. 6). This fragment may arise via cleavage of the α-carbon-carbon bond of the lysine moiety of the diester, followed by the loss of the elements of alanine ethyl ester.

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The absence of a peak at 102 ($-CH(NH_2)COOC_2H_5$) excludes a primary α-amino ester[6] whereas the presence of a peak at mass 30 suggests a $-CH_2NH_2$ group. Furthermore, the amino and ester groups can not be in a 1,6 relationship to each other, because it was found that in such a case (ornithine) only the spectrum of the corresponding lactam is obtained[6].

All this evidence is most compatible with the structure (I). The only alternative formula, having the $-NH_2$ group at the γ-carbon is eliminated because of the virtual absence of a peak at mass 58 ($CH_3-CH_2-CH-NH_2$) which would have to be observed instead of mass 30.

Structure (I) has now been confirmed by synthesis. N-ε-carbobenzoxy-L-lysine was condensed with D, L-α-bromopropionic acid in presence of $Ba(OH)_2$[7].

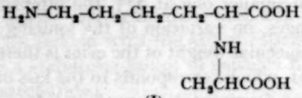
After decarbobenzoxylation of the resulting condensation product and purification on Amberlite IRC-50 a crystalline compound was obtained, needles softening at about 160° with decomposition at 240°, $[\alpha]_D + 16 \pm 1°$ in water (*c*, 1.1). (Found: C, 46.29; H, 8.92; N, 11.57. $C_8H_{18}N_2O_4 \cdot H_2O$, requires C, 45.75; H, 8.53; N, 11.86).

This substance was identical with the natural amino acid by the following criteria: R_F values in 3 solvents; electrophoresis at 900 V at pH 3.6 and pH 6.5; retention volume on a Moore and Stein column; infrared spectrum (in Nujol) and finally the mass spectrum of the diethyl ester.

The synthetic compound is probably still a mixture of diastereo-isomers, having the α-carbon of the alanine moiety respectively in the D and L configuration. We hope to report soon the exact stereochemistry of the natural amino acid.

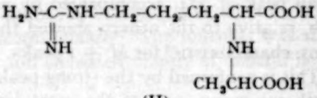
We propose the name *lysopine* for the new amino acid (I) as it is the lysine analogue of octopine (II), a characteristic component of octopus muscle.

We are indebted to Dr. S. Moore (New York) for an analysis of the natural amino acid on one of his automatic columns and to Dr. P. Jollès (Paris) for the comparison of the natural and synthetic samples by high-voltage electrophoresis and column chromatography, and to Dr. E. Bricas (Paris) for the samples of ε-N-carbobenzoxy-L-lysine.



$H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\underset{\underset{\underset{CH_3CHCOOH}{|}}{NH}}{\underset{|}{CH}}\text{-}COOH$

(I)
Lysopine



$H_2N\text{-}\underset{\underset{NH}{\|}}{C}\text{-}NH\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\underset{\underset{\underset{CH_3CHCOOH}{|}}{NH}}{\underset{|}{CH}}\text{-}COOH$

(II)
Octopine

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Received March 23rd, 1960

Reduction of 5′-cytidylic acid to deoxycytidylic acid by mammalian enzymes

This report describes the conversion of ^{14}C-labeled CMP to deoxycytidine nucleotides by a cell-free extract of a rat tissue, the Novikoff ascites tumor. It has been demonstrated previously (see Reichard[1]) that *in vivo* and in living-cell systems cytidine can be incorporated into DNA deoxycytidine with the glycosidic bond intact. Small conversions of ribonucleosides and ribonucleotides to the deoxyribose derivatives have been obtained by Grossman[2,3] in bacterial extracts and by Reichard[1] in chick-embryo extracts. The conversion has not been obtained previously in extracts of mammalian tissues.

The tumor cells were ruptured by homogenization in 1.5 vol. of distilled water and centrifuged at 100,000 × *g* to obtain the supernatant fraction. Dialysis of the extracts increased the enzyme activity. $HClO_4$ extracts were made of the incubation mixtures, carrier dCMP was added, and the extracts were heated to hydrolyze polyphosphates. The dCMP was re-isolated by chromatographic procedures, on Dowex-50 and paper, based on the methods of Reichard[4]. The amount of [^{14}C]dCMP formed was calculated from the amount of carrier dCMP added and the specific radioactivity of the isolated dCMP.

Table I illustrates the requirements for ATP, TPN, and a reducing system in the reaction. In the complete system, [^{14}C]CMP (prepared from [6-^{14}C]orotic acid[5]) was phosphorylated and maintained as cytidine triphosphate; in the absence of ATP, the CMP was destroyed with consequent low conversion to dCMP. TPN was clearly required and the activity was augmented by a TPN-reducing system, although a DPN-lactate system was nearly as effective. The reaction rate was constant for 2 h of incubation but decreased subsequently.

Added [^{14}C]cytidine was rapidly phosphorylated by the enzyme extract, and was as effective a substrate as [^{14}C]CMP. When the substrate was "uniformly-labeled" [^{14}C]cytidine (Schwarz Laboratories, Inc.), with a ratio of specific activities (base/nu-

TABLE I

SUBSTRATE REQUIREMENTS FOR THE CONVERSION OF [^{14}C]CMP TO DEOXYCYTIDINE NUCLEOTIDES BY NOVIKOFF-TUMOR EXTRACT

The complete system contained, in a final volume of 1.5 ml: 25 μmoles Tris buffer, pH 7.0; 11.2 μmoles $MgCl_2$; 7.5 μmoles ATP; 0.6 μmole TPN; 6.0 μmoles glucose 6-phosphate; 0.39 μmole [^{14}C]CMP, 37,500 counts/min; 0.1 unit of commercial glucose-6-phosphate dehydrogenase; and 1.0 ml of enzyme extract (dialyzed for 12 h against 0.02 *M* Tris buffer, pH 6.5 at 4°). Sodium lactate replaced glucose 6-phosphate in No. 6.

Conditions	*Counts/min in dCMP*
1. Complete system	2100
2. 2.5 μmoles ATP instead of 7.5 μmoles	500
3. ATP omitted	5
4. Glucose 6-phosphate omitted	1600
5. TPN omitted	20
6. DPN instead of TPN	1700

Abbreviations: CMP, cytidine 5′-phosphate; dCMP, 2′-deoxycytidine 5′-phosphate; Tris, tris(hydroxymethyl)aminomethane hydrochloride; gluc-6-P, glucose 6-phosphate; ATP, adenosine triphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; DNA, deoxyribonucleic acid.

cleoside) of 0.68, it was found that the ratio of the product dCMP was 0.66. This indicated that the deoxyribose of the dCMP was derived from the ribose of the substrate. The radioactivity of deoxycytidine (isolated with carrier) was negligible with either [^{14}C]CMP or [^{14}C]cytidine as substrate.

Centrifugation of the enzyme extract, adjusted to pH 5 with acetic acid, yielded "pH-5 soluble" and "pH-5 precipitate" fractions, both of which were found to be necessary for the overall reaction. Both fractions were labile to heat and stable to dialysis. As shown in Table II, the sequence and substrate requirements of the two enzyme fractions were explored. The action of the pH-5 soluble enzyme with the TPN-reducing system and possibly with ATP was necessary before or simultaneously with the incubation of the pH-5 precipitate with [^{14}C]CMP. The pH-5 soluble fraction appears to produce an essential, unidentified component, while the pH-5 precipitate fraction appears to catalyze the reduction of CMP nucleotides.

TABLE II

SEQUENCE OF ACTION OF THE TWO FRACTIONS

After the first incubation for 1 h, the tubes were heated 2 min in a boiling-water bath, cooled and the additional substrates and enzyme were added for the second 1-h incubation. The final amounts of components were the same as in Table I.

Components in first incubation		*Additional components in second incubation*		*Counts/min in dCMP*
enzyme preparation	*substrates*	*enzyme preparation*	*substrates*	
1. pH-5 precipitate	ATP, MgCl$_2$, TPN, Gluc-6-P, [^{14}C]CMP	pH-5 soluble	—	0
2. pH-5 soluble	—	pH-5 precipitate	ATP, MgCl$_2$, TPN, Gluc-6-P, [^{14}C]CMP	20
3. pH-5 soluble	ATP, MgCl$_2$, [^{14}C]CMP	pH-5 precipitate	TPN, Gluc-6-P	120
4. pH-5 soluble	ATP, MgCl$_2$, TPN, Gluc-6-P, [^{14}C]CMP	pH-5 precipitate	—	270
5. pH-5 soluble	TPN, Gluc-6-P	pH-5 precipitate	ATP, MgCl$_2$, [^{14}C]CMP	270
6. pH-5 soluble	ATP, MgCl$_2$, TPN Gluc-6-P	pH-5 precipitate	[^{14}C]CMP	410
7. pH-5 precipitate + pH-5 soluble	ATP, MgCl$_2$, TPN Gluc-6-P, [^{14}C]CMP	(no second incubation)		710
8. whole extract	ATP, MgCl$_2$, TPN, Gluc-6-P, [^{14}C]CMP	(no second incubation)		790

These experiments support the concept that the deoxyribose of DNA deoxycytidine may be derived from the ribose of cytidine by a reductive step at the nucleotide level.

This work was supported by U.S.P.H.S. grant NCI-C-4464.

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Received February 23rd, 1960

Electrophoresis of nucleic acids in silica gel

The use of freshly prepared silica gel as an anticonvection medium in zone electrophoresis was reported orginally by Consden, Gordon and Martin[1] who used these gels in the electrophoretic fractionation of amino acids and peptides. Gordon *et al.*[2] observed that proteins would not migrate in silica gel, apparently because the pores of the gel were too small to permit movement of the large protein molecules. This laboratory has reported recently a method for the rapid preparation of silica gels containing appropriate electrophoretic buffers and their use in zone electrophoresis[3]. This note reports the results of some attempts to carry out electrophoretic fractionations of RNA and DNA by electrophoresis in silica gel.

Crude RNA from yeast was prepared by the method of Crestfield, Smith and Allen[4] and was fractionated by the use of 1 *M* NaCl. Both the NaCl-soluble and -insoluble fractions were precipitated with cold ethanol and washed with 65 % ethanol until free of NaCl. They were then dissolved in water and adjusted to pH 7. DNA was prepared by the method of Hurst[5] and was freed of a small amount of contaminating protein by shaking with chloroform and amyl alcohol[6]. The nucleic acids were subjected to electrophoresis in silica gel at pH 7.0 using triethanolamine buffer of 0.1 ionic strength. The silica gel was prepared as described previously[3]. The NaCl-soluble RNA moved rapidly as a narrow band in the silica medium. The NaCl-insoluble RNA, on the other hand, exhibited varying degrees of mobility in the gel. A portion of this fraction migrated as rapidly as the soluble RNA and the remainder showed lesser mobilities in varying degrees, the slowest material having about one-fourth the mobility of the fastest. The channels containing the two fractions were cut in 1-cm segments following electrophoresis, the RNA eluted with 0.1 *M* HCl, and the absorbancies read in the spectrophotometer at 260 mμ. The results are given in Fig. 1. Samples of the leading portion, center, and trailing portion of the NaCl-insoluble RNA were taken and resubmitted to electrophoresis in separate inlays, and it was found that each sample migrated as before. When the three samples were mixed and resubmitted to electrophoresis from a single inlay, three bands were obtained with mobilities similar to the starting materials.

DNA, in contrast to RNA, did not migrate in the gel but remained bound at the site of application. Experiments to determine how readily nucleic acids could

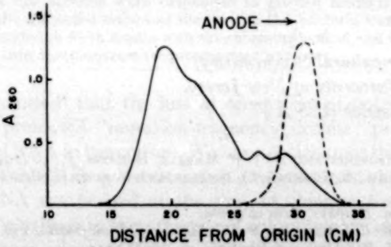

Fig. 1. Electrophoretic migration of NaCl-soluble RNA and NaCl-insoluble RNA in silica gel. Conditions: 15 V/cm for 4.3 h; triethanolamine (acetate) buffer, pH 7.0, *I* 0.1. – – –, NaCl-soluble RNA; ——, NaCl-insoluble RNA.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

be extracted from silica gel were carried out as follows: solutions of soluble and insoluble RNA, and DNA, in 0.1 M phosphate buffer at pH 7 were mixed separately with equal volumes of silica sol (6 % in SiO_2) and allowed to gel. Each gel was then homogenized with 5 vol. water in a Potter-Elvehjem glass homogenizer for 1 min. The gel residue was centrifuged down and the nucleic acid content in the supernatant determined. 98 % of the NaCl-soluble RNA, 60 % of the NaCl-insoluble RNA, and about 1 % of the DNA were extracted from the gel by this procedure. As shown in Table I, extremes of pH are required for extraction of DNA from the gel. At pH 1 destruction of the DNA occurs, while at pH 9.2 or 10.2 extraction of the DNA is probably facilitated by changes in the gel structure.

TABLE I

EXTRACTUON OF DNA FROM SILICA GEL

Final concentration of DNA in the gel prior to extraction is 0.1 %. In all procedures the volume of extracting solution used is five times the volume of the undried gel.

Extraction procedure	*DNA in supernatant (% of total A_{260} units)*
Homogenization with water	0.5–2
Homogenization with 2 M NaCl	2
Gel dried *in vacuo*, 0°:	
first homogenization with water	1.8
second homogenization with water	0.5
third homogenization with water	0.2
Gel dried with acetone–ether, homogenized with water	0
Gel held at pH 10.2, 0.5 h, 20°	92
Gel held at pH 9.2, 0.5 h, 20°	~53
Gel held at pH 1.0, 1 h, 100°	93

Freshly prepared silica gel may prove to be useful in the fractionation of RNA, separation of RNA from DNA and protein, or in other procedures involving the separation of compounds of low and high molecular weights. The ability of peptides and RNA to migrate electrophoretically in the gel while proteins and DNA remain bound at the origin suggests that the gel acts as a molecular sieve which slows or prevents the movement of large molecules while allowing small molecules to migrate without hindrance.

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Received February 8th, 1960

Chloramphenicol, nucleic acid synthesis and mutation induced by ultraviolet light

Several recent findings[1,2] suggest that DNA synthesis is the terminal event in u.v.-induced mutation in bacteria. Mutation expression follows closely the initial synthesis of DNA in the culture following u.v. exposure. Further, other studies[3-6] have shown that post-irradiation treatments blocking RNA or protein synthesis (*i.e.* incubation with 6-aza-uracil or chloramphenicol) are effective in lowering markedly the mutation-frequency response to ultraviolet light. Various investigators[7-9] have demonstrated that blockage of RNA or protein synthesis delays the recovery of DNA synthesis in u.v.-irradiated bacteria. It has recently been suggested by LIEB[10] that chloramphenicol reduces mutation frequency in bacteria by delaying DNA synthesis.

This question was investigated through studies of u.v.-induced reversion to prototrophy of the tryptophan-requiring auxotroph, *Escherichia coli* strain WP2. The techniques used have been previously described[5,6].

Table I demonstrates that when chloramphenicol is added to the incubating culture immediately after irradiation there is a reduction in the expected induced mutation frequency to a minimal level within 20 min. However, this treatment has no effect on subsequent DNA synthesis in the culture. Therefore, the contention[10] that chloramphenicol causes a lowered mutation-frequency response through delay of DNA synthesis does not appear plausible. It appears more reasonable that blockage of protein synthesis promotes an *active* "mutation-frequency decline" process as previously suggested by us[5,6].

TABLE I

EFFECT OF CHLORAMPHENICOL ON MUTATION FREQUENCY AND ON SUBSEQUENT DNA SYNTHESIS

*Time (min) with chloramphenicol**	*Prototrophs per 10^8 surviving auxotrophs (75 min)***	*Relative amount DNA with incubation (min)*			
		45	*60*	*75*	*90*
0	112	1.1	1.5	2.1	2.7
20	20	1.1	1.5	2.1	2.6

* After u.v. exposure the bacteria were suspended in growth medium containing 20 μg/ml chloramphenicol. After the indicated period of incubation the bacteria were rapidly centrifuged out of this media, resuspended in fresh media without chloramphenicol and incubation continued.

** Between 20 and 80 min the mutation frequency does not vary.

We have demonstrated[6] that the loss of sensitivity of potential mutations to the chloramphenicol promoted "mutation-frequency decline" process is correlated with initial doubling of RNA in the culture. We have designated[5] this loss of sensitivity "mutation fixation". Table II demonstrates that a relation exists between the amount of post-irradiation RNA synthesized at the time of chloramphenicol addition, the relative rate of DNA synthesis in the presence of chloramphenicol and the mutation-frequency response observed upon plating. Recovery of the capacity to synthesize DNA after u.v. exposure is related to the initial post-irradiation doubling of RNA in the culture. Apparently, the necessary RNA and protein must be formed prior

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; u.v., ultraviolet light.

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to chloramphenicol addition in order for DNA to be replicated with consequent establishment of mutation in the genome. Where this mandatory RNA and protein synthesis has not occurred prior to chloramphenicol addition the potentiality for mutation is lost, presumably through the active decline process.

TABLE II

RELATION BETWEEN "MUTATION FIXATION", THE AMOUNT OF RNA SYNTHESIZED PRIOR TO CHLORAMPHENICOL ADDITION AND SUBSEQUENT RATE OF DNA SYNTHESIS IN THE PRESENCE OF CHLORAMPHENICOL

Time of chloramphenicol addition (min)*	*Mutation fixation** %*	*RNA*** increase in %*	*DNA synthesis****				
			relative amount with incubation (min)				*% of maximum rate in chloramphenicol*
			60	*70*	*80*	*90*	
0	16.7	0	1.06	1.02	1.08	1.00	0
30	22.2	17	1.12	1.19	1.24	1.33	20
40	32.9	36	1.18	1.24	1.43	1.53	40
50	61.3	63	1.18	1.38	1.58	1.77	62
60	93.6	102	1.23	1.55	1.87	2.06	97

* After u.v. exposure the cells were diluted into growth media and incubated. Chloramphenicol (20 μg/ml) was added at the indicated time. All times measured from start of incubation following u.v. exposure.

** Mutation frequency after incubation for 45 min in chloramphenicol (a period of time sufficient to eliminate all chloramphenicol-sensitive mutations) expressed as per cent of maximum frequency.

*** DNA synthesis in the presence of chloramphenicol. RNA formed prior to chloramphenicol addition.

Blockage of DNA synthesis during the "chloramphenicol challenge" period has no effect on the mutation frequency observed[11]. This finding is confirmed by use of 6-aza-uracil instead of chloramphenicol as the "mutation-challenging" agent. 6-aza-uracil blocks net RNA, protein and DNA synthesis but gives an identical "mutation-fixation" curve to that demonstrated by chloramphenicol[12]. Thus the hypothesis recently presented by LIEB[10] that the amount of DNA synthesis occurring in the presence of chloramphenicol in 60 min is significant in determining "mutation fixation" appears to be without validity. It is evident that the acquisition of the *capacity* to synthesize DNA rather than DNA synthesis is involved in "mutation fixation". Following u.v. exposure, the synthesis of RNA and protein endows the bacterium with the capacity both for DNA synthesis and mutation induction. The nature of the function of the RNA and protein in genetic replication and mutational change remains to be determined.

This work was supported in part by a grant from the U.S. National Institutes of Health and by U.S. Atomic Energy Commission Contract AT-(40-1)-2139.

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Received March 8th, 1960

6-Hydroxylation, the major metabolic pathway for melatonin

LERNER *et al.*[1–4] have demonstrated the presence of a melanocyte-contracting substance, melatonin (N-acetyl-5-methoxytryptamine), in bovine pineal glands and in the peripheral nerves of man, monkey, and cattle. AXELROD AND WEISSBACH[5] found a methyl transferase in pineal glands which can O-methylate N-acetylserotonin to melatonin. Little is known about the metabolic fate of this hormone in the body. Although LERNER *et al.*[6] have demonstrated 5-methoxyindole-acetic acid in bovine pineal glands, the origin of this compound is uncertain.

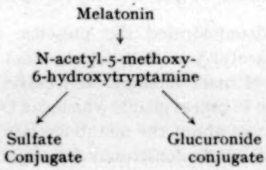
In order to study the metabolic fate of this hormone, [2-^{14}C]melatonin was synthesized from [2-^{14}C]serotonin, and administered intraperitoneally to albino male rats and the urine collected. Chromatography of the urine on Whatman No. 1 paper in isopropanol–5 % ammonia (8:2) and subsequent scanning for radioactive peaks indicated the presence of at least three radioactive compounds ($R_F = 0.14$, 0.54 and 0.75) the second peak ($R_F = 0.54$) containing about 80 % of the excreted radioactivity. Only two peaks ($R_F = 0.25$, 0.80) were found in butanol–acetic acid–water (4:1:1). None of the peaks had R_F's corresponding to melatonin, 5-methoxytryptamine, or 5-methoxyindoleacetic acid. A similar distribution of radioactivity was found on chromatography of the urine of rats which had received [^{14}C-*methoxy*]-melatonin or [^{3}H-*acetyl*]melatonin intraperitoneally indicating that the major metabolites retained both the 5-methoxy and the N-acetyl groups.

After the administration of a large dose of melatonin (20 mg), chromatography of the urine and spraying with Ehrlich's reagent indicated the presence of two indolic compounds, having the same R_F's as the first two radioactive metabolites in the isopropanol–ammonia system ($R_F = 0.14$, 0.54). These observations indicated that the α-position of the indole ring was unsubstituted. Hydrolysis of the eluted major component with a sulfatase preparation (Gluculase, Endo Products, N.Y.) in the presence of an antioxidant (ascorbic acid) or under N_2 resulted in the formation of a compound having a different R_F in butanol–acetic acid–water (4:1:1) [$R_F = 0.67$]. No change in R_F followed treatment with bacterial β-glucuronidase (Sigma Chemical Co., St. Louis). The hydrolysed compound reacted with diazotized *p*-nitroaniline to form a purple-colored derivative indicating the presence of a free phenolic group. Both hydrolysed and unhydrolysed metabolites gave red-colored compounds with acidic diazotized sulfanilic acid, the compound containing the free phenolic group reacting more rapidly. This reaction is characteristic of 6-hydroxyindoles and their sulfates[7,8]. Additional evidence for the identity of this metabolite as N-acetyl-5-

methoxy-6-hydroxytryptamine was obtained by treating melatonin with the model oxidizing system[9] which has been shown to hydroxylate indoles on the 6-position[10]. Incubation of melatonin with this system resulted in the formation of a compound having the same R_F's and color reactions as the major excretion product of melatonin after hydrolysis.

The indolic compound having an R_F of 0.14 in isopropanol–5 % ammonia (8:2) was also isolated and shown to have an R_F of 0.11 in the butanol–acetic acid–water system. Hydrolysis of this compound with β-glucuronidase resulted in the formation of two indolic compounds, one having the same R_F and color reactions as the compound tentatively identified as N-acetyl-5-methoxy-6-hydroxytryptamine.

These observations indicate that the principle pathway of metabolism of melatonin is as follows:



The methods of preparation of the radioactive compounds used will be reported later.

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Received March 24th, 1960

The isolation of a new complex lipid: triphosphoinositide from ox brain

By solvent fractionation FOLCH[1] prepared from ox brain a phospholipid which he designated diphosphoinositide and which consisted in its simplest composition of one molecule each of inositol, glycerol and fatty acid together with two molecules of phosphoric acid. After short acid hydrolysis its main phosphorus-containing product was found to be inositol-*m*-diphosphate. Recently a similar diphosphoinositide fraction from brain was shown to yield three spots on a paper chromatogram[2].

FOLCH AND LEBARON[3-5] also described a complex inositol-containing phosphatido-peptide which was extracted from lipid-free trypsin-digested brain material with acidified solvent. This contained phosphorus and inositol in the molar ratio 2.7:1, a sphingosine-like substance, fatty acid, and abundant nitrogen, mainly in the form of peptide-linked amino acids; it yielded inositol diphosphate on acid hydrolysis.

Recently a new hydrolytic technique[6] has been developed for the quantitative examination of the phospholipids in a complex lipid fraction isolated from tissues. When this method was applied to lipid extracted from brain tissue by chloroform–methanol (2:1, v/v) it failed unexpectedly to give evidence for the presence of any inositol-containing phospholipid apart from monophosphoinositide. The reverse was true, however, if the tissue was pre-treated with acetone prior to the chloroform-methanol extraction. This observation has been made the basis of a method for isolating the complex inositide fraction from ox brain, the purification being followed by preparing acid and alkaline hydrolysates of the fractions and examining these by high-voltage ionophoresis and chromatography.

The brain tissue is exhaustively extracted with chloroform–methanol to remove all other lipids, and the residue then extracted with slightly acidified chloroform–methanol solvent[4]. When the latter extract is shaken with aq. 0.9% NaCl and centrifuged, the inositide is largely found in the interfacial protein layer. After heating this with acetone and ethanol, the protein is all removed and the chloroform-soluble inositide is obtained. On adding methanol, a methanol-insoluble fraction is precipitated, designated triphosphoinositide A. If the supernatant is now neutralized with methanolic NaOH, the main component of the fraction, triphosphoinositide B, is precipitated as its sodium salt.

The sodium salt of triphosphoinositide B is soluble in water and chloroform but insoluble in methanol. It is virtually nitrogen-free. Its simplest composition is found to be (fatty acid)$_6$(phosphate)$_6$(glycerol)$_3$(inositol)$_2$ and this accounts for all of the components of the molecule. After brief acid hydrolysis it yields considerable diglyceride together with free glycerol, fatty acids, and a trace of free inositol. Of the water-soluble phosphorus-containing components formed, inositol triphosphate has been isolated in a yield of 75–80%; a compound containing (inositol)$_1$-(phosphate)$_2$, a few per cent of glycerophosphoric acid and traces of inorganic phosphorus comprise the rest of the hydrolysate. The fatty acids on examination by gas chromatography have been shown to be a complex mixture with stearic acid, arachidonic acid and a C_{22} polyunsaturated acid predominating; palmitic, oleic, linoleic acids and another C_{20} unsaturated acid are also present.

A fraction equivalent in composition to the triphosphoinositide A fraction is obtained in much better yield, by working up the lipids obtained by chloroform-methanol extraction of acetone-pretreated brain tissue. The Folch I fraction[7] prepared from such an extract is run through an Amberlite IRC 120 column which removes cations and most of the contaminating inorganic phosphate. The lipid is then distributed in a biphasic system formed from chloroform, ether, ethanol and water. The product, thus separated from contaminating phosphatidyl serine and monophosphoinositide, is insoluble in methanol. Analysis indicates the simplest composition as $(\text{glycerol})_1$-$(\text{inositol})_1$ $(\text{phosphate})_3(\text{acyl ester})_{2?}$. On brief acid hydrolysis, mono- and diglycerides can be isolated from the hydrolysate. The phosphorus-containing hydrolysis products are very similar to those from triphosphoinositide B except that more inorganic P and less inositol triphosphate are formed. After mild alkaline hydrolysis[6] the predominant phosphorus-containing product analyses as $(\text{glycerol})_1(\text{inositol})_1(\text{phosphate})_3$.

By means of alumina and silicic acid columns[8,9], monophosphoinositide giving the correct analysis has also been obtained from brain lipid extracts. This confirms the recent evidence, obtained by chromatography, for the existence of this lipid in brain tissue[2,6,9].

The present results indicate that as well as monophosphoinositide, brain tissue contains two triphosphoinositides which are probably very closely related in structure, and which are tightly attached to brain proteins. No information has yet been obtained regarding the relation of these to diphosphoinositide or the relative amounts of the various inositol-containing lipids.

Dr. D. RHODES is thanked for the fatty acid analysis of triphosphoinositide B. One of us, J. C. DITTMER, is a U.S. Public Health Service Research Fellow of the National Heart Institute.

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Received March 25th, 1960

Biochim. Biophys. Acta, 40 (1960) 379–380

A mechanism of *orthol*-hydroxylation of aromatic amines *in vivo*

Several groups[1–3] have obtained data with various species which suggest that separate enzyme systems catalyze the *p*-hydroxylation and *o*-hydroxylation of aromatic amines and amides *in vivo*. It was recently found in this laboratory that N-hydroxy-

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2-acetylaminofluorene is a major metabolite in the rat of the carcinogen 2-acetylaminofluorene[4]. Subsequent studies[5] on the metabolic fate of this new metabolite have shown that while it yields the same urinary metabolites given by 2-acetylaminofluorene[6] it gives rise to 3 to 4 times as much 1-hydroxy-2-acetylaminofluorene and less of the other phenolic metabolites than does the parent amide. This suggested that the N-hydroxy derivative may rearrange *in vivo* to form the 1-hydroxy derivative, possibly in a manner analogous to the rearrangement of arylhydroxylamines to phenolic amines in acid[7]. Data in support of this hypothesis are presented herein.

Adult male albino rats, 8-weeks old, were fed 0.03 % of 2-acetylaminofluorene in a purified diet for 6 weeks to produce the liver changes which insure the excretion of the N-hydroxy derivative when the parent amide is fed[5]. The rats were then fed the purified diet alone for 6 days to permit metabolites of the amide to disappear from the urine. Each rat was fed a mixture (equimolar) of 0.97 mg [9-^{14}C]-2-acetylaminofluorene ($2 \cdot 10^6$ counts/min/μmole) and 1.04 mg of unlabeled N-hydroxy-2-acetylaminofluorene in 1 g diet. The urine was collected for the next 6 h. Following the procedures of WEISBURGER *et al.*[8] the urinary metabolites were released enzymically from their conjugates, extracted with ether, and separated chromatographically on a silicic acid column with cyclohexane–*tert.*-butanol–acetic acid–water (18:2:2:1, v/v/v/v). This yielded satisfactory samples of the N-, 5-, and 7-hydroxy derivatives of 2-acetylaminofluorene. A band containing a mixture of the N-, 1-, and 3-hydroxy derivatives was resolved by descension chromatography on Whatman No. 1 paper with the above solvent mixture after the paper had been thoroughly extracted with this solvent mixture. The compounds were eluted from the paper with ethanol. The absorbancy ratios of the spectra of the various eluates were in good agreement with the corresponding values for the authentic derivatives measured under the same conditions. The eluates were plated onto aluminum planchets from ammoniacal ethanol solutions and the radioactivities were determined in a gas-flow end-window Geiger counter. As shown in Table I the 1-hydroxy derivative had only about one-fourth the specific activity of the other phenolic metabolites. The specific activities of the urinary 2-acetylaminofluorene and N-hydroxy-2-acetylaminofluorene are consistent with the fact that these compounds are interconvertible to some extent *in vivo*[5]. Previous work[5] has also shown that the precursor of all the hydroxy derivatives is a compound which retains the acetyl group of the administered parent amide.

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN URINARY METABOLITES AFTER ADMINISTRATION OF A MIXTURE OF [9^{14}C]-2-ACETYLAMINOFLUORENE AND UNLABELED N-HYDROXY-2-ACETYLAMINOFLUORENE TO RATS

Specific activity of administered [9^{14}C]-2-acetylaminofluorene = $2 \cdot 10^6$ counts/min/μmole.

Urinary metabolite	*Specific activity (counts/min/μmole)*
2-Acetylaminofluorene	$0.78 \cdot 10^6$
N-hydroxy-2-acetylaminofluorene	$0.60 \cdot 10^6$
1-hydroxy-2-acetylaminofluorene	$0.40 \cdot 10^6$
3-hydroxy-2-acetylaminofluorene	$1.2 \cdot 10^6$
5-hydroxy-2-acetylaminofluorene	$1.7 \cdot 10^6$
7-hydroxy-2-acetylaminofluorene	$1.8 \cdot 10^6$

These results demonstrate that in the rat the 1-hydroxy metabolite of 2-acetylaminofluorene arises largely, if not entirely, from the N-hydroxy metabolite, whereas the 5- and 7-hydroxy derivatives come largely, if not exclusively, from the parent amide. The intermediate activity of the 3-hydroxy metabolite indicates that some of this *o*-hydroxy derivative is derived from the N-hydroxy metabolite. Thus, while hydroxylation at the *para* site (7-) and certain of the *ortho* sites (3-, 5-) apparently involves a direct attack at these ring positions by activated oxygen[9], hydroxylation of the other *ortho* site (1-) apparently involves the intermediary formation and rearrangement of the N-hydroxy derivative, probably via the corresponding *o*-quinolimide (cf. ref. 5).

This investigation was supported by Grant C355 of the National Cancer Institute, U.S. Public Health Service, a grant from the Jane Coffin Childs Memorial Fund for Medical Research, and the Alexander and Margaret Stewart Trust Fund. We are grateful to Mrs. JOANNE PIERINGER for valuable technical assistance.

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Received March 25th, 1960

Biochim. Biophys. Acta, 40 (1960) 380–382

A specific synthetic polypeptide antigen

All the specific antigens described till now are either natural substances (proteins, polysaccharides) or are derived from them by chemical modification. The large number of reported failures to detect antibodies against various synthetic homopolymers of α-amino acids suggests that the polymers studied are at the most very weak antigens[1]. Out of tens of synthetic polypeptides investigated by STAHMANN *et al.*[2,3] only one particular sample of poly-L-glutamic acid and a multichain copolymer of glutamic acid, leucine, glycine and lysine were reported to be antigenic. The antibodies formed did not precipitate with the homologous synthetic polypeptides, but cross-reacted with related polypeptidyl albumins as well as with various unrelated proteins. The synthetic polypeptides did not inhibit precipitin reactions between their antisera and proteins. MAURER *et al.*[4] reported recently that antibodies to a linear copolymer of glutamic acid and lysine could be detected by passive cutaneous anaphylaxis, but not by the precipitin reaction. Only tobacco-mosaic-virus protein inhibited the anaphylaxis reaction.

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An investigation of the antigenicity of various polypeptidyl gelatins showed that, among others, the attachment of copolymeric chains of tyrosine and glutamic acid converted gelatin into a strong antigen whose specificity was due entirely to the polypeptides attached[5,6]. The question arises in this case whether gelatin still contributes in any way towards the immunological properties of the new antigen. In view of the above we have considered the possibility of replacing the gelatin with a synthetic material.

We have now been able to demonstrate that a multichain copolymer, in which chains of polypeptides containing L-tyrosine and L-glutamic acid were built on a multichain poly-DL-alanine[7,8] is a powerful and sharply specific antigen in rabbits. This copolymer was obtained by the polymerization in dioxane-0.05 M phosphate buffer (pH 7) (1:4), of N-carboxy-L-tyrosine anhydride and γ-benzyl-N-carboxy-L-glutamate anhydride, using the α-amino groups of multi-poly-DL-alanyl-poly-L-lysine[7,8] as polymerization initiators, followed by debenzylation with anhydrous hydrogen bromide; mol. wt. = 180.000; 9.9 % L-tyrosine residues; 15.3 % L-glutamic acid residues; 67.5 % DL-alanine residues; 7.0 % L-lysine residues.

The synthetic material was injected three times, at fortnightly intervals, in a complete adjuvant mixture into rabbits. The antibodies formed could be demonstrated by the usual precipitin reaction (Table I and Fig. 1). In order to determine directly the antigen in the specific precipitate, a preparation labelled with ^{131}I was used for precipitation. Except for the homologous antigen, the antibodies could be cross-precipitated only with the related copoly (tyrosyl, glutamyl) gelatin (Fig. 1). The homologous reaction could be inhibited completely by a copolymer of L-tyrosine and L-glutamic acid in a residue molar ratio of 1:1, only partially by a copolymer of these amino acids in a ratio of 1:4, and not at all by a copolymer of the same amino acids in a ratio of 1:9. Neither cross-reaction nor inhibition was obtained with linear poly-DL-alanine, multichain poly-L-glutamic acid, or unrelated proteins such as egg albumin or human γ-globulin.

Multichain poly-DL-alanine (the material which yielded the synthetic antigen after enrichment with tyrosine and glutamic acid), as well as a linear copolymer of L-glutamic acid and L-tyrosine in a residue molar ratio of 1:1 (degree of polymerization, 31), failed to produce antibodies detectable by the homologous precipitin test

TABLE I

COMPOSITION OF THE IMMUNOSPECIFIC PRECIPITATES

Antigen added per 1 ml serum (µg)	*Antigen precipitated* (µg)*	*Antigen precipitated (%)*	*Antibody precipitated** (µg)*	*Antibody/antigen in precipitate (w/w)*
12.5	12.35	99.0	195	15.4
31.2	26.5	85.0	321	12.1
62.5	47.2	75.5	418	8.9
125	70.0	56.0	468	6.7
250	80.8	32.3	412	5.1
437	79.3	18.2	345	4.3
750	77.5	10.4	260	3.4
1250	70.3	5.6	212	3.0

* From radioactivity data.
** From extinction at 2800 Å of neutralized solutions of the precipitates in NaOH, after deducting the calculated extinction of the antigen.

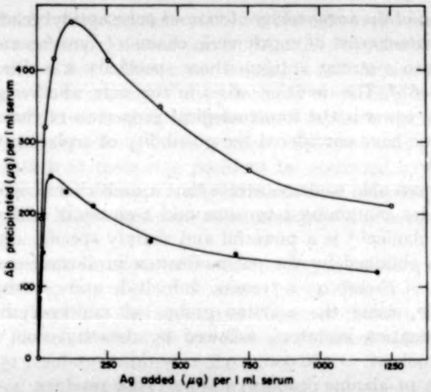

Fig. 1. Precipitin curves of copoly (tyrosyl, glutamyl) multichain poly-DL-alanine (○) and copoly (tyrosyl, glutamyl) gelatin (●) with the antiserum to copoly (tyrosyl, glutamyl) multichain poly-DL-alanine. The amounts of antibodies were obtained from extinction at 2800 Å after deducting the calculated extinction of the antigens. The amount of antigen in the precipitate was obtained for the synthetic material from the radioactivity data, and for the gelatin derivative from hydroxyproline data.

when injected similarly into rabbits. Nor could antibodies be detected by cross-precipitation with related polypeptidyl derivatives.

In view of the enhancement of the antigenicity of gelatin by attachment of tyrosine and other aromatic amino acids[5,6], and in view of the strong specific antigenicity of the tyrosinated synthetic multichain polypeptide, it seems that the potentiation of antigenicity by tyrosination may be of a more general character.

A study, now in progress, of the possible antigenicity of various other synthetic linear and multichain copolymers of α-amino acids should permit the elucidation of the role of molecular weight, shape, composition, and locus of specific amino acids, in conferring the antigenic properties on a molecule.

This investigation was supported by grant A-3083 of the National Institutes of Health, U.S. Public Health Service.

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Received March 22nd, 1960

THE RELATIONSHIP BETWEEN INACTIVATION OF TOBACCO MOSAIC VIRUS BY X-RAYS AND BREAKAGE OF NUCLEIC ACID*

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(Received October 1st, 1959)

SUMMARY

The analytical ultracentrifuge, with u.v. absorption optics, has been used to demonstrate and measure bond breakage in the main chain of RNA strands extracted from X-irradiated TMV.

When the whole virus was irradiated in solution at 0°, the rate of loss of intact RNA strands was about equal to the rate of loss of viral activity. However, the reduction in yield of extracted RNA that accompanied increasing X-ray dose, together with the probability that longer strands would be selectively lost, cast considerable doubt on the significance of this result.

When TMV was irradiated while frozen at about —50° or while in centrifugal pellets with little water present, the correlation between reduction in yield and X-ray dose disappeared. These data indicate a D_0 for strand breakage of $4.0 \cdot 10^5$ R as compared to an average measured D_0 of $1.8 \cdot 10^5$ R for virus inactivation. Thus, breakage proceeds 40% as rapidly as does inactivation. Also, 40% of all the ionizations in TMV RNA induced by radiation may be expected to occur among backbone chain electrons. If it is assumed[1] that any ionization within the nucleic acid complement of a virus inactivates the virus, then the present data indicate that ionizations within the backbone chain of TMV RNA break main chain bonds and cleave the strand with an efficiency of close to 100%. By analogy, then, it is suggested that ionizations elsewhere in the nucleic acid also are effective in breaking bonds.

INTRODUCTION

There is good evidence that absorption of ionizing radiation within the tobacco mosaic virus (TMV) particle inactivates by inducing damage in the nucleic acid (RNA) moiety. EPSTEIN[1] and BUZZELL, TRKULA AND LAUFFER[2] have calculated the X-ray sensitive volume of TMV to be about equal to its RNA volume. GIERER AND SCHRAMM[3] and FRAENKEL-CONRAT, SINGER AND WILLIAMS[4] have demonstrated TMV activity in solutions of practically protein free RNA. GINOZA AND NORMAN[5] have determined the X-ray inactivation rate of infectious RNA to be the same as that of intact TMV. The physical nature of the lethal change remains to be determined.

LAUFFER, TRKULA AND BUZZELL have shown that both X-ray[6] and u.v.[7] irradiation of whole TMV decrease the intrinsic viscosity of subsequently extracted RNA

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[*] Publication No. 70 of the Department of Biophysics.

strands. They suggested that both the viscosity decrease and inactivation of the virus might be due to a radiation induced breakage of the virus' RNA strands. The present communication reports further investigations of the physical effect on RNA of X-irradiation of TMV. Using the Spinco analytical ultracentrifuge equipped with u.v. absorption optics, we have been able to resolve whole from somewhat degraded RNA, to measure the relative concentrations of the two, and thus to follow the kinetics of an X-ray induced degradation of intact nucleic acid strands.

METHODS AND MATERIALS

The type strain of tobacco mosaic virus was prepared from systematically infected Turkish tobacco plants by the method of BOEDTKER AND SIMMONS[8,9], but with fewer high speed centrifugations.

Virus solutions were sprayed at low pressure in microdrops onto collodion covered grids for electron microscopic examination.

RNA was isolated from the virus by SCHRAMM's phenol method[3] modified by limiting the six extractions to 1 min each as suggested by BAWDEN AND PIRIE[10].

X-radiation with a mean wave length of 0.2 Å, as determined by absorption in copper, was delivered by a 200 kV Westinghouse deep therapy unit. To reduce the effectiveness of radiation induced free radicals of water[11], inactivation experiments were performed at concentrations of 1 % to 2 % of virus, either in 10 % Difco nutrient broth solution or frozen in buffer at about —50°. For the various breakage experiments, TMV was irradiated at 1° and at —50° in buffer and at 1° in wet pellets obtained by centrifuging from neutral, 0.001 *M* Versene solution at 35,000 × *g* for 90 min.

U.v. absorption spectra of nucleic acid solutions were measured in the Beckman DU spectrophotometer. Maxima and minima were found at 257–258 mμ and 230–231 mμ, respectively. RNA concentration was found by dividing O.D. at 257 mμ by the factor 25.3 $(\text{mg/ml})^{-1}$ (ref. 12). Virus concentrations were determined with a differential refractometer by use of the factor 0.0017 $(\text{mg/100 ml})^{-1}$ (ref. 13). Relative nucleic acid yields were calculated by assuming the virus to be 6 % by weight RNA.

For the analytical centrifugations, conditions were so controlled that the spinning rotor reached a temperature of about 4° when full speed was attained. The rotor temperature drifted no more than several tenths of a degree during the runs. Samples were run at an O.D. at 257 mμ of 1.00 to 1.25, corresponding to concentrations of 0.004 % to 0.005 % for nucleic acid, which was centrifuged at 59,780 rev./min, and to 0.04 % for TMV, which was centrifuged at 25,980 rev./min. Pictures of the absorption profile of the spinning cell were obtained on Kodak "commercial" film (Fig. 1). A picture was taken during acceleration (frame 1), at speed (frame 2), and every 4 min thereafter (frames 3–10). Exposure time was 40 sec. Films were developed in half strength Kodak DK 50 developer at 20° for 2 min with continuous brushing. Photodensitometric tracings were made with a suitably adapted Beckman analytrol adjusted by use of calibrated neutral density filters to read true density of the films.

Viscosities were measured at 4.0° with a Cannon-Manning semi-micro viscometer having an average velocity gradient of 300 sec^{-1}.

Optical activity of RNA solutions was measured with a Rudolph model 80 precision polarimeter at wavelengths 546 mμ and 589 mμ.

Viral activity was assayed by the local lesion method using *Nicotiana glutinosa*. Unknowns were inoculated in parallel with standard samples at several dilutions on the plan of a Latin square so that each sample was inoculated once on each plant and once on each leaf position. Activities of unknowns were expressed in terms of concentration of standard sample necessary to produce an equal number of lesions.

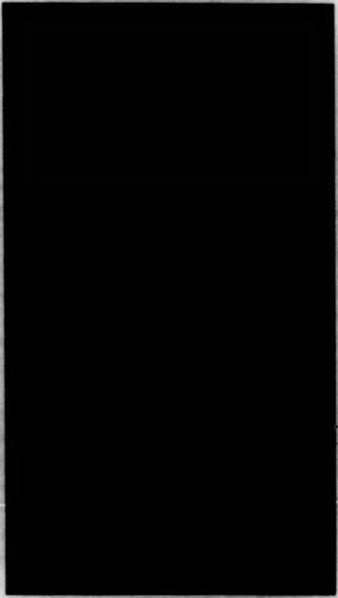

Fig. 1. Positive print of pictures obtained with the u.v. absorption optics of the analytical ultracentrifuge showing the sedimentation pattern of unirradiated RNA.

The inactivation dose (D_0) was estimated from a straight line plot of logarithm of activity remaining *versus* the X-ray dose.

The solvent employed throughout was 0.02 M phosphate buffer at pH 7.2 unless otherwise specified.

Analysis of the ultracentrifuge data

A set of pictures obtained from unirradiated RNA is reproduced in Fig. 1. The two outer areas provide reference lines at known distances from the rotor axis; the inner oblong represents the cell itself. From left to right can be distinguished the air space above the meniscus, the meniscus as a sharp line, a non-absorbing region of solvent from which all the molecules have migrated, a region of increasing absorption indicating particles moving within a range of velocities, an abrupt increase in absorption indicating a major fraction of particles moving with uniform velocity, and a uniform region extending to the bottom of the cell. Superimposed densitometric

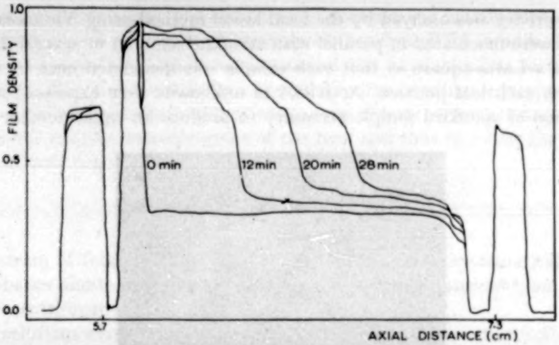

Fig. 2. Superimposed photodensitometric tracings of frames 1, 5, 7 and 9 of Fig. 1.

traces of frames 1, 5, 7 and 9 are presented in Fig. 2. The leading component moves with a sedimentation coefficient corrected to water at 20°, S_{20}^{w}, of 31.

Traces were analyzed to determine the relative amounts of RNA sedimenting as the leading component and as the hetero-dispersed, slower component. With the conditions of film treatment previously described and the RNA concentration here employed, the trace height was approximately proportional to concentration. Corrections were made when necessary for non-linearity at the very top and bottom of the traces. Correction was also made for the dilution experienced by all sedimenting components[14]. Traces 1, 5, 7 and 9 (Fig. 2) were used in the analysis of each centrifuge run. From frames 5, 7 and 9 was taken the average trace height for the region of the cell containing buffer only. The difference between this height and the height of trace 1 was taken to indicate undiluted, total RNA concentration in the cell. The corrected heights corresponding to the leading component and to the trailing component were expressed in terms of percent of the total. The use of this measure renders the analysis independent of dilution errors in previous handling of the samples and of differences in film development among the various runs. Estimations from the three traces of the fraction of the total in each component were averaged.

RESULTS

Data concerning unirradiated virus and RNA extracted from it are presented in Table IA. A number of points are noteworthy.

Half of the parent virus sedimented with a leading component with S_{20}^{w} of 190. This is the full length monomer[15] and the slower material can be only broken rods which normally occur in TMV preparations. The sedimentation pattern for RNA from unirradiated virus approximates that of the virus. Thus the RNA moving with the leading component is derived from full length virus rods while the more slowly sedimenting strands are derived from broken rods. It appears probable on the basis of hydrodynamic data that the entire RNA complement of the virus sediments in one piece as the leading component. The S_{20}^{w} of this component is 31. Intrinsic viscosity characteristic of the component is estimated to be about 200 ml/ml when allowance

TABLE IA

UNIRRADIATED SAMPLES

Sample	*Extraction yield (%)*	*Absorption maximum/minimum*	*Fraction in main component (%*
Parent TMV I			51
TMV I, frozen and thawed			49
RNA from TMV I	95	2.56	46
	79	2.36	45
	86	2.15	44
RNA from TMV II pellet	100	2.44	75

TABLE IB

IRRADIATED SAMPLES

Sample	*Dose ($R\cdot10^5$)*	*Extraction yield (%)*	*Absorption maximum/minimum*	*Fraction in main component (%)*
RNA from TMV I irradiated in solution	0.39	72	2.28	37
	1.2	66	2.22	22
	1.6	72	2.08	19
	2.1	59	2.02	15
	3.5	49	2.14	10
RNA from TMV I irradiated frozen	0.78	81	2.20	30
	1.8	80	2.16	36
	2.7	85	2.29	23
	3.7	76	2.07	24
	5.5	69	2.09	16
RNA from TMV II irradiated in pellets	1.0	88	2.14	52
	1.9	85	2.20	40
	2.8	84	2.03	37

is made for the viscosity contribution of the broken strands. Taking the partial specific volume as 0.57[16] and using the equation of MANDELKERN AND FLORY[17] for a random coil, one obtains a mol. wt. of $2\cdot10^6$ if the effective hydrodynamic unit is assumed to be spherical. These data and calculations agree with those recently published by BOEDTKER[18] for heat extracted TMV RNA and by GIERER[19–21] for phenol extracted TMV RNA. Both authors have concluded that the leading component corresponds to the whole RNA complement of TMV.

Data characterizing RNA extracted from irradiated virus are summarized in Table IB. In Fig. 3 is given a representative series of photodensitometric traces showing comparable sedimentation patterns for RNA extracted from virus exposed to different doses of X-rays. These data show that when parent virus is subjected to increasing doses of X-irradiation more and more of the RNA appears in the trailing component at the expense of the leading component. This is thought to reflect breakage of nucleic acid strands.

Fig. 4 is a semi-log plot of the relative fraction of RNA remaining in the leading component after various doses of irradiation. The plot indicates that RNA breakage proceeds as a first order, single event reaction. The 37 % dose, D_0, for breakage of RNA extracted from TMV which has been X-irradiated in solution at 0° is $1.8 \cdot 10^5$ R. Several determinations of the D_0 for inactivation of the parent TMV irradiated under the same conditions (except for inclusion of 10 % broth in the virus solution) gave the values $1.6 \cdot 10^5$ R, $2.0 \cdot 10^5$ R and $2.4 \cdot 10^5$ R, so that apparent RNA breakage and

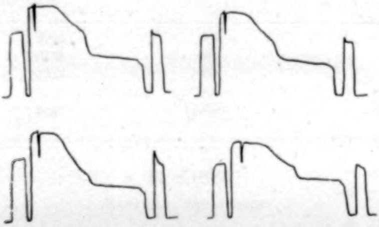

Fig. 3. Tracings after 20 min of sedimentation (frame 7) for RNA extracted from virus receiving various radiation treatments: upper left unirradiated; upper right $0.39 \cdot 10^5$ R in solution; lower left $1.56 \cdot 10^5$ R in solution; lower right $1.75 \cdot 10^5$ R, frozen at —50°.

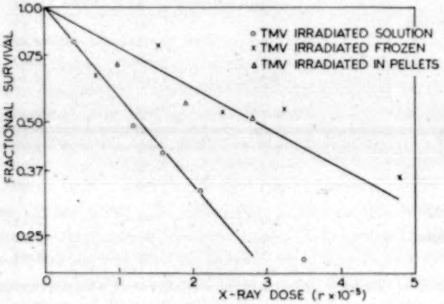

Fig. 4. Semi-log plot of fraction of intact RNA to survive breakage after various X-ray doses.

loss of viral activity proceed at essentially the same rate. However, the persistent decrease in the yield of extracted RNA with increasing X-ray dose makes the significance of this result questionable. A selective loss of longer strands could obscure the true breakage rate. When the virus was irradiated while frozen at about —50° or in a wet pellet the radiation induced reduction in yield was essentially eliminated (Table IB). These data are plotted in Fig. 4 and indicate a D_0 for RNA breakage of $4.0 \cdot 10^5$ R. The D_0 for inactivation of frozen virus was measured at $1.2 \cdot 10^5$ R and $1.6 \cdot 10^5$ R in two experiments.

DISCUSSION

Evaluation of the method

The difficulties associated with the analytical ultracentrifugation of low concentration solutes have received considerable attention. The question is whether sedimenting solute particles can create a density gradient in the cell sufficient to withstand the mixing tendencies of mechanical shock and local temperature fluctuation encountered during the run. SCHUMAKER AND SCHACHMAN[22] and SHOOTER AND BUTLER[23] have been able to perform evidently convection free centrifugations of desoxyribonucleic acid preparations at concentrations as low as 0.02 mg/ml. With the conditions of centrifugation previously described we have found a rather high degree of reproducibility for the measurements we have made. No run of this project showed disturbances in the principle boundary indicative of significant convection. The possibility of convection below and/or above the boundary, however, cannot be excluded. Indeed the JOHNSTON-OGSTON effect[24] might be expected to evoke convection above the boundary. The low concentrations used make it seem unlikely that the error in concentration measurement deriving from this effect[25] would here be of significant size.

Electron micrographs of virus stock I showed that about 50% by wt. of the virus particles were the uniform, 3000 Å monomer. The ultracentrifugal analysis estimated this value at 50%. Three runs of unirradiated RNA (stock I, three independent extractions) gave values of 45%, 46% and 47% in the leading component. Eight runs of a somewhat different sample (five independent extractions) gave values between 38% and 41% in the forward boundary. Thus the method can measure reproducibly the fraction of intact RNA in the solutions of interest in this study.

Breakage of bonds in TMV RNA

X-irradiation of the whole virus decreased the sedimentation coefficient of some of the extracted RNA previously in the leading edge. Intrinsic viscosity was concurrently decreased. These observations constitute good evidence for scission of RNA strands. The alternative possibility of an extensive change in shape and hydration is ruled out also by the observation that no change in specific optical rotation of the RNA occurred even after ten times the D_0 dose.

In the RNA extraction procedure, the aqueous TMV solution is shaken with phenol and then centrifuged to bring down the phenol rich phase and denatured virus protein. RNA strands incompletely separated from their denatured protein matrices during the shaking operation might well be lost in centrifugation and it seems likely that longer strands would be selectively removed. When TMV was irradiated in solution, extraction losses of RNA increased with increasing X-ray dose (Table IB). Therefore the very interesting result, that RNA breakage and virus inactivation proceeded at nearly the same rate, is suspect. It seems probable that the decrease in fraction of whole strands observed in the ultracentrifugal analysis was due both to breakage and to selective loss during extraction.

That the decrease in RNA yield was due to chemical attack on the protein by X-ray induced free radicals of water was indicated by the experiments involving the irradiation of TMV in the frozen state and in centrifuged pellets with very little water present. Here the yield was largely independent of dose and the ultracentrifugal analysis should measure the true rate of RNA breakage. The data indicate a D_0 for RNA breakage of $4.0 \cdot 10^5$ R as compared to an average value of $1.8 \cdot 10^5$ R for virus inactivation. The rate of main chain breakage, then, is about 40% of the rate of virus inactivation. From the reported base ratios in TMV RNA[26–28] and the known primary structure of RNA, the fraction of electrons that are associated with atoms in the main backbone chain can be calculated. The number is close to 40% independent of whether only bonding electrons are considered or whether all the electrons are included.

If it is assumed, following EPSTEIN[1], that any ionization within the RNA moiety inactivates the virus, then the present data indicate that ionizations associated with main chain atoms lead to breaks in main chain bonds with an efficiency of about 100%, when the RNA is irradiated while enclosed in its protein matrix. Since ionizations break main chain bonds with high efficiency, it is likely that ionizations elsewhere in the nucleic acid also are effective in breaking bonds and this, therefore, becomes a plausible explanation for X-ray inactivation.

ACKNOWLEDGEMENTS

We are indebted to J. A. WOHLHIETER for virus stocks used in the experiments reported here and for aid in characterizing the preparations.

This research was supported by contract No. AT(301)913, Atomic Energy Commission. S.W.E. held Public Health Service pre-doctoral research fellowship No. CF6637 from the National Cancer Institute.

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VIRUS PARTICLE ADSORPTION

II. ADSORPTION OF VACCINIA AND FOWL PLAGUE VIRUSES TO CELLS IN SUSPENSION

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(Received September 7th, 1959)

SUMMARY

The rates of adsorption of radioactively-labelled fowl plague and vaccinia viruses to fowl red blood cells, chick embryo cells, HeLa cells and ascites tumour cells in suspension have been studied. The observed rates were less than the calculated rates of collision between virus particles and cells. The initial rates of adsorption corresponded to those expected if one third of collisions between virus particles and cells is effective for adsorption. Later the rates of adsorption fell, mainly owing to clumping of the cells. The rates of adsorption were the same to cells which support virus multiplication and those which do not. Elution of fowl plague virus from fowl red blood cells at 37° was rapid, but very little elution of fowl plague or vaccinia viruses took place from tissue culture cells at 37°.

INTRODUCTION

In the first paper of this series[1] equations were derived to define the rate of collision between small particles, such as viruses, and larger spherical objects, such as cells, suspended in a fluid medium. Reasons were given why the approximate forms of the equations that can be used to calculate the collision rates between phage particles and host bacteria are not applicable to animal viruses and their host cells.

In this paper the adsorption of radioactively labelled viruses to a variety of host cells is described. Some of the host cells used were capable of supporting the multiplication of the viruses, others were not. In all cases, however, the rates of attachment of virus particles to cells were of the same order, which was several times less than that expected from Brownian theory. This stands in contrast to the rates of attachment of viruses to glass and other non-biological surfaces which under optimal conditions are very close to those expected from Brownian theory[1]. In a later paper[2] the rates of attachment of viruses to cell monolayers will be described, and the effects of some variables on adsorption analysed. These results suggest reasons why the adsorption of viruses to cells is less efficient than adsorption to aluminium, glass, nitrocellulose and carbon surfaces. They are also of practical use in calculating the proportion of virus particles adsorbed in experiments on virus multiplication.

MATERIALS AND METHODS

Virus

The preparation of purified ^{131}I-labelled vaccinia virus and ^{32}P-labelled fowl plague virus, and techniques used for assaying radioactivity, have been described previously[1].

Red blood cells

The red blood cells used were obtained from adult fowl blood collected in acid–citrate–dextrose, centrifuged, the buffy coat removed and the cells washed twice in normal saline. The cells used were not agglutinated by vaccinia.

Ascites tumour cells

S 37 sarcoma ascites tumour cells were passaged at 10 day intervals in C3H mice. For experimental purposes they were harvested after 8 days, washed twice in Gey's solution and resuspended by vigorous pipetting. They were allowed to stand for 1 min in a siliconized tube and the uppermost cells used.

HeLa cells

HeLa cells were propagated in the following growth medium: 70 parts of Gey's solution; 10 parts of 5 % lactalbumin hydrolysate; 10 parts of 1 % yeast extract; 10 parts of human serum. Cultures were washed with calcium- and magnesium-free saline (Gey's solution A) and incubated for 15 min at 37° in phosphate buffered saline containing 0.05 % trypsin. The liberated cells were washed twice in Gey's solution and resuspended by vigorous pipetting in the adsorbing medium. They were allowed to stand for 1 min in a siliconized tube and the uppermost layer of cells were used.

Chick embryo cells

Petri dish cultures of cells from 10 day old chick embryos were prepared by a modification[3] of DULBECCO's technique. The cultures were incubated at 37° in growth medium until a confluent layer of cells was obtained. The cultures were washed, trypsinized and again washed and resuspended as described for the HeLa cells.

Cell counts and measurements

Cell counts were made in a haemocytometer, 1000 cells being counted in each case, which reduces the random error of the count to less than 4 %. Cell diameters were measured in wet suspensions by a Filar micrometer and by measurement of photomicrographs. Agreement of the results by the two techniques was satisfactory. With each cell type distributions of radii were plotted and the geometric mean was taken for calculations.

THEORETICAL

It has been shown[1] that the maximum fraction of virus particles that can be adsorbed by a suspension of cells, *i.e.* when every collision between a particle and a cell leads to adsorption, is given by

$$f = 1 - \exp\{-4\pi nRD(t + \mathrm{R}\sqrt{t/\pi D})\} \qquad (1)$$

where n is the number of cells of radius R, t is the time and D is the diffusion coefficient of the virus. D can be calculated from the STOKES-EINSTEIN equation,

$$D = \frac{kT}{6\pi\eta a} \tag{2}$$

where k is BOLTZMANN's constant, T the absolute temperature, η the viscosity of the suspending fluid and a the radius of the particles.

The experiments to be reported below show that the observed rate of adsorption of virus is considerably less than that expected from equation (1). When only a fraction of the collisions is successful (*i.e.* leads to adsorption), an exact mathematical treatment of this situation is excessively difficult. In the early stages of adsorption, however, the form of the equation will be similar to the above, but with a factor ϕ equal to the proportion of successful collisions multiplying the exponential term, *i.e.*

$$f = 1 - \exp\{-4\phi\pi nRD(t + R\sqrt{t/\pi D})\} \tag{3}$$

Where the proportion of successful collisions is very small (small values of ϕ), this equation will be very approximate except for a short period after commencing adsorption. However, if $\phi \geqslant {}^1/_3$, as we have found it to be in most cases of animal virus adsorption, equation (3) appears to be closely followed for periods at least up to 30 min, when other factors, notably clumping of cells, significantly alter the adsorption rate.

EXPERIMENTAL

Eight tubes containing 1.9 ml washed red cells in Gey's solution without bicarbonate, buffered to pH 7.0 with 0.02 M sodium phosphate were prepared, four with 10^7 cells/ml and four with $5 \cdot 10^6$ cells/ml. At zero time 0.1 ml ^{32}P-labelled fowl plague virus was added to the cell suspension, which was resuspended by shaking every 7.5 min. At 7.5 min, 15 min, 30 min and 60 min the cells were chilled and centrifuged, the supernatant removed and the cells washed twice in cold Gey's solution. Samples of the supernatant, and volumes of the resuspended red cells, were dried down on 1 cm^2 planchettes for assay of radioactivity in an end-window counter. The proportion of the original count associated with the cell fraction was taken as the proportion of virus adsorbed after various time intervals. In most experiments this result was 4 to 7% lower than the count expected by subtraction of the final count in the supernatant from the original count. This small discrepancy was due partly to loss of virus during washing, and partly to elution of virus from the cells; this takes place to some extent even in the cold (see below).

The results are shown in Fig. 1. Curve A shows the maximum rate of adsorption calculated from equation (1) on the assumption that every collision between virus particles and cells results in adsorption. Curve B is calculated on the assumption that the fraction of effective collisions is one third. The observed rate of attachment of virus to cells is given in curve C, which initially coincides with curve B. When about 60% of the virus is adsorbed, however, the observed rate of adsorption falls. That this was not due to the presence of a proportion of virus particles less easily adsorbed than the rest was shown in experiments in which the virus remaining in the supernatant after 15 min was added to another suspension of cells. The rate of adsorption was then similar to that initially observed. An important factor con-

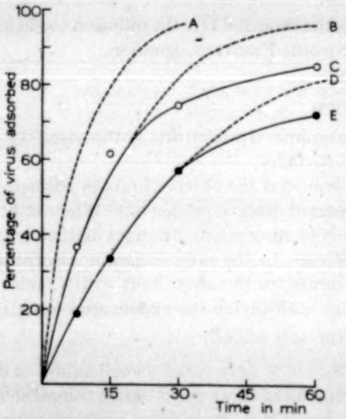

Fig. 1. Adsorption of labelled fowl plague virus by fowl red blood cells in suspension. A – theoretical maximum adsorption rate for 10^7 cells/ml. B – adsorption rate calculated for 10^7 cells/ml assuming one third of collisions are effective. C – observed adsorption rate by 10 cells/ml. D – the adsorption rate calculated for $5 \cdot 10^6$ cells with one third of collisions effective. E – observed adsorption rate for $5 \cdot 10^6$ cells.

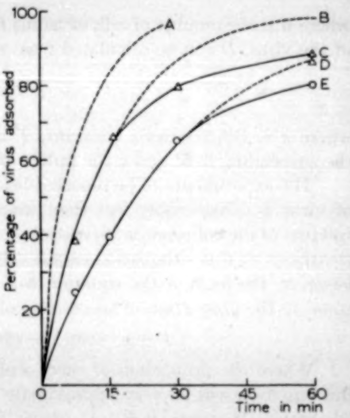

Fig. 2. Adsorption of labelled fowl plague virus by chick embryo cells in suspension. A – theoretical maximum adsorption rate for 10^7 cells/ml. B – adsorption rate calculated for 10^7 cells/ml assuming one third of collisions are effective. C – observed adsorption rate by 10^7 cells/ml. D – the adsorption rate calculated for $5 \cdot 10^6$ cells with one third of collisions effective. E – observed adsorption rate for $5 \cdot 10^6$ cells.

tributing to the fall in adsorption rate is clumping of the cells; this was evident microscopically after about 15 min under these conditions. This reduces the cell area available for virus attachment. The fact that some elution of virus occurs even at 20° (see below) could also be contributory.

Adsorption of fowl plague virus by suspensions of chick embryo cells and HeLa cells

Suspensions of 10^7 and $5 \cdot 10^6$ chick embryo cells/ml were prepared in buffered Gey's solution as described in the section on METHODS. The rate of adsorption of ^{32}P-labelled fowl plague virus was measured as for red cells. The results are shown in Fig. 2. The initial adsorption rate again coincides with that expected on the assumption that one third of the surface area is effective. The rate of adsorption by HeLa cells, in which this strain of fowl plague grows poorly, was also of the same order of magnitude (Fig. 3).

Adsorption of vaccinia virus by HeLa cell suspensions

The rate of adsorption of vaccinia virus to HeLa cell suspensions is also shown in Fig. 3. The rate is lower than that of fowl plague virus, by the factor expected from the difference in diffusion coefficients of the two viruses. Again, the initial adsorption rate coincided with the expectation from equation 1 on the assumption that one third of the collisions are effective.

Adsorption of vaccinia virus by ascites tumour cells in suspension

The rate of adsorption of labelled vaccinia virus to ascites tumour cells in sus-

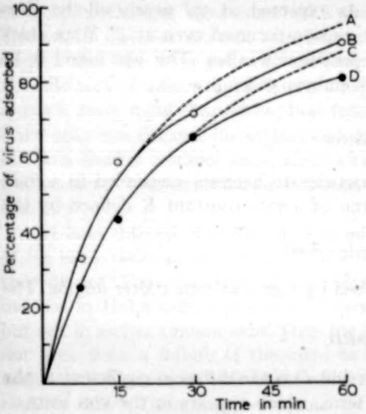

Fig. 3. Adsorption of labelled fowl plague and vaccinia viruses by HeLa cells in suspension A – adsorption rate calculated for fowl plague virus by $5 \cdot 10^6$ HeLa cells/ml on the assumption that one third of collisions are effective. B – observed adsorption rate of fowl plague virus by $5 \cdot 10^6$ HeLa cells/ml. C – adsorption rate calculated for vaccinia virus by $5 \cdot 10^6$ HeLa cells in suspension assuming that one third of collisions are effective. D – observed adsorption rate of vaccinia virus by $5 \cdot 10^6$ HeLa cells/ml.

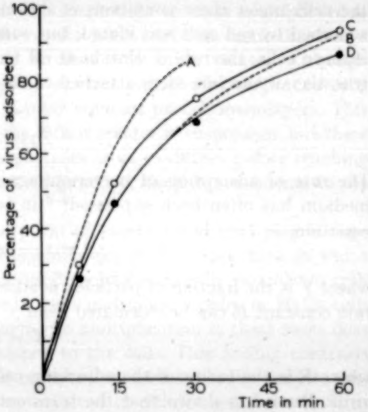

Fig. 4. Adsorption of labelled vaccinia virus by ascites tumour cells in suspension. A – adsorption rate calculated for vaccinia virus by $5 \cdot 10^6$ ascites tumour cells/ml assuming one half of collisions are effective. B – the same assuming that one third of collisions are effective. C – observed rate of adsorption of vaccinia virus by $5 \cdot 10^6$ ascites tumour cells/ml. D – observed rate of adsorption of vaccinia virus by $5 \cdot 10^6$ trypsin-treated ascites tumour cells/ml.

pension is shown in Fig. 4. The rate was actually somewhat higher than that expected on the assumption that one-third of the collisions with the cells result in adsorption, but it fell to almost exactly this figure after treatment of the cells with trypsin.

Elution of virus from cells

The elution of labelled fowl plague virus from chick embryo cells, HeLa cells and fowl red blood cells was investigated. Cells to which virus had been adsorbed at room temperature were washed in cold Gey's solution and resuspended in Gey's solution without bicarbonate buffered with 0.02 *M* sodium phosphate at pH 7.0. Large volumes were used to reduce readsorption of eluted virus by cells. After 1 h at 4°, 20° and 37° the cells were centrifuged and radioactivity (representing virus still associated with cells) was counted. In Table I the proportion of virus eluted from

TABLE I

PERCENTAGE OF ^{32}P-LABELLED FOWL PLAGUE VIRUS ORIGINALLY ATTACHED TO CELLS ELUTED AT VARIOUS TEMPERATURES (pH 7.0)

Temperature	*0°*	*20°*	*37°*
Fowl red cells	8	20	95
Chick embryo cells	4	4	7

the cells under these conditions is shown. As expected, at 37° nearly all the virus adsorbed by red cells was eluted, but some elution occurred even at 4°. With chick embryo cells, the rate of elution at all temperatures was low. This was found to be true also of vaccinia virus attached to chick embryo cells.

DISCUSSION

The rate of adsorption of bacteriophage particles to bacteria suspended in a fluid medium has often been expressed[4-7] in terms of a rate constant K defined by the equation

$$f = 1 - \exp(-Knt)$$

where f is the fraction of particles n adsorbed by bacilli at time t after mixing. The rate constant K can be calculated from

$$K = 4\pi RD$$

where R is the radius of the adsorbing cells and D is the diffusion coefficient of the virus. PUCK has shown that the transient term, which appears in the VON SMOLUCHOWSKI formulation, can be neglected for all practical purposes in this system.

Attempts have, however, been made to use the same equations to describe the adsorption of animal viruses to suspensions of red blood cells[9] and tissue culture cells[9-11] which have radii of 4 to 10 μ. As previously pointed out[1], in these systems K will not be a constant until about 1 h after adsorption starts. A more exact formulation is given in equation (3).

The main finding in our experiments has been that the two dissimilar animal viruses studied, vaccinia and fowl plague, are adsorbed by cells in suspension at rates considerably below those expected from the calculated rates of collision between virus particles and cells. Initially, adsorption occurs at rates which suggest that one third of the collisions between virus particles and cells are effective for adsorption. Later the rates of adsorption fall, mainly because of clumping of the cells, which reduces their effective surface area.

It seems clear that the efficiency of virus adsorption relative to collision frequency is somewhat higher in monolayers of cells (one half of collisions effective[2]) than in trypsinized suspensions of the same cells (one third of collisions effective). This suggests that trypsinization removes from the cell surface a protein component which not only promotes intercellular adhesion, but also facilitates virus adsorption, perhaps because it alters the charge of the cell surface. There is evidence that trypsinization of S 37 ascites tumour cells produces a significant loss of dry mass measured by interferometry[12], and enzyme treatment of ascites tumour cells changes their electrophoretic mobility[13]. Measurements of electrophoretic mobility of red cells show differences from white cells[14], and it is interesting that, so far as efficiency of virus adsorption is concerned, untreated red cells should behave like trypsinized tissue culture cells.

However, an obvious difference between red cells and tissue cultures shows up in elution. Fowl plague virus is rapidly eluted from red cells at 37°, but only slowly from chick embryo cells, in accordance with previous observations on Newcastle disease[9]. The quantitative studies of adsorption here reported are useful from the practical point of view, since they enable reasonably accurate calculations to be made of the proportion of virus adsorbed by cells in experiments on virus multiplication. The results of other workers with herpes B[15], poliovirus[11] and Newcastle disease[9,10] suggest that the same order of efficiency is attained with other viruses. Although the efficiency of adsorption is lower, adsorption of virus particles by suspended cells is much more rapid than adsorption from similar volumes by cell monolayers. This is not only true because the surface area of the cells is greater in suspension, but there is also a smaller distance over which virus particles have to diffuse before reaching cell surfaces. This expectation is borne out in practice, despite statements to the contrary[9].

It is of interest that the rates of adsorption of vaccinia and fowl plague were of the same order in cells in which the viruses multiply readily and those in which they do not. Thus the strain of fowl plague used multiplies rapidly in embryo cells but not in HeLa cells; the strain of vaccinia used multiplies rapidly in HeLa cells but not in ascites tumour cells. Thus the barrier to multiplication in these cases does not arise from a failure of the virus to adsorb to the cells. This finding contrasts with the behaviour of poliovirus, which is reported to adsorb readily only to susceptible cells[16]. There may be various factors involved in cellular resistance to virus infection.

ACKNOWLEDGEMENTS

We are indebted to Dr. H. G. PEREIRA for help in preparing ^{32}P-labelled fowl plague virus, and to Drs. C. KAPLAN and A. S. MCFARLANE for supplying and iodinating the vaccinia virus.

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VIRUS PARTICLE ADSORPTION

III. ADSORPTION OF VIRUSES BY CELL MONOLAYERS AND EFFECTS OF SOME VARIABLES ON ADSORPTION

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(Received September 7th, 1959)

SUMMARY

The rates of adsorption of ^{32}P-labelled fowl plague virus and ^{131}I-labelled vaccinia virus by monolayers of chick embryo and HeLa cells have been investigated. With both types of virus and cell the observed rates of adsorption were very nearly one half the rates expected from Brownian theory and observed when virus was adsorbed by glass and other non-biological surfaces. The adsorption of viruses by cells is not depressed by protein. Adsorption of viruses by cells is dependent on the concentration of cations in the medium, and is depressed by high concentrations of multivalent cations. The rate of adsorption is unaffected by heat inactivation, weak formalin treatment and thiol reagents. The rate of adsorption is increased by low concentrations of polycations and in the presence of high concentrations of uranyl ions; it is diminished by low concentrations of polyanions and by acetylation of the amino groups of the virus. It is suggested that the main interacting groups are the amino groups of the virus and phosphate groups of the host cell wall. The effects of ions on adsorption cannot be explained by current theories describing the interaction of charged surfaces.

INTRODUCTION

In the first paper of this series[1] equations were developed from the theory of Brownian motion defining the rate of arrival of particles in suspension at a surface. The actual rates at which virus and other particles of similar size are adsorbed by nitrocellulose, glass, carbon, gold and aluminium surfaces were investigated. It was shown that, even when the suspensions were agitated, under optimal conditions the proportions of particles adsorbed agreed closely with theoretical expectation.

In the second paper of the series[2] the kinetics of adsorption of viruses by suspensions of red blood cells, ascites tumour cells and tissue culture cells were analysed. The rates of adsorption were found to be appreciably lower than theoretical expectation from the calculated frequency of collisions between virus particles and cells. The rates of adsorption of radioactively labelled vaccinia and fowl plague viruses by cell monolayers are described in the present paper. Again, the observed adsorption is significantly less than that expected from Brownian theory, although the deficiency is not as marked as with cells in suspension. In an attempt to explain the less efficient adsorption of viruses by cells than by non-biological surfaces and to define some of the factors influencing adsorption of animal viruses to cells, the conditions of adsorption, charge of adsorbing surfaces (ionic strength, hydrogen ion concentration, and temperature) have been varied, and the results of these experiments are also outlined and discussed.

MATERIALS AND METHODS

Radioactively labelled virus

The preparation of purified vaccinia virus labelled with ^{131}I and fowl plague virus labelled with ^{32}P, and the techniques of measuring radioactivity, were previously described[1].

Cell monolayers

Monolayers of chick embryo tissues in 5-cm and 6-cm diameter Petri dishes were prepared by the method of DULBECCO[3]. In some experiments HeLa cell monolayers were prepared by seeding the Petri dishes with $5 \cdot 10^7$ cells in 5 ml growth medium (70 parts Gey's solution; 10 parts human serum; 10 parts of 1 % yeast extract in Gey's solution; and 10 parts 5 % lactalbumin hydrolysate in Gey's solution), and the cells incubated until they formed a uniform sheet on the bottom of the Petri dish.

Adsorption was measured by adding 0.5 to 2 ml of labelled virus in the appropriate medium to the washed cell monolayer, leaving for various times of adsorption and terminating the process by rapidly removing the supernatant fluid and washing the cell monolayer repeatedly with the same medium but no virus. Appropriate controls showed that there was no significant elution of virus from the cells during the course of washing or preparation for counting.

The cells were suspended by incubation with 0.1 % trypsin in Gey's solution and the radioactivity of the cell suspension measured. For ^{131}I counts, the cell suspension was made up to 2 ml, placed in standard tubes and counted in a well-type scintillation counter. For ^{32}P counts the cell suspension (evenly spread with a small quantity of detergent) was dried down onto 1-cm^2 planchettes and assayed in a Geiger end-window counter.

THEORETICAL

It has been deduced[1] that the number, N, of particles suspended in fluid reaching a unit area of flat surface in contact with the fluid is given, for less than 40 % of the particles adsorbed, by

$$N = 11.3\, c_0 \sqrt{Dt} \tag{1}$$

where c_0 is the initial concentration of particles in the fluid (number/unit volume), D is the diffusion coefficient of the virus and t is the time.

For more than 28 % of the particles adsorbed,

$$N = c_0 d \left(1 - \frac{8}{\pi} \exp\{-\pi^2 Dt/4d^2\}\right) \tag{2}$$

where d is the depth of fluid above the surface.

The diffusion coefficient of the virus, D, can be calculated from the STOKES-EINSTEIN equation:

$$D = \frac{kT}{6\pi\eta a} \tag{3}$$

where k is BOLTZMANN's constant ($1.38 \cdot 10^{-16}$), T is the absolute temperature, η the viscosity of the suspending fluid and a the radius of the particles.

Previous experiments[1] showed that, when virus and polystyrene latex particles suspended in fluid in the neighbourhood of neutrality are adsorbed by surfaces of nitrocellulose, glass, carbon and gold, the observed rate of adsorption is close to expectation from Brownian theory (equations 1 and 2), provided the concentration of cations is sufficient. The same is true of adsorption by positively charged aluminium surfaces even in the absence of ions.

This result follows from the net negative charge of the virus above pH 5.6 which, allows adsorption by positively charged surfaces under a wide range of conditions. Adsorption by negatively charged surfaces, on the other hand, can only take place when the concentration of cations is sufficient to reduce the thickness of the ionic double layer on the two surfaces, which can then come close enough together for short-range attractive forces to bring about adsorption.

The presence of even small amounts of protein strongly depresses attachment of virus particles to non-biological surfaces. It was suggested[1] that the rapidly diffusing protein soon becomes adsorbed on to the surface, conferring on it hydrophilic properties and so hindering the attachment of virus or polystyrene latex particles.

EXPERIMENTAL

Rate of attachment of virus to cell monolayers

Experiments were carried out with ^{32}P-labelled fowl-plague virus and ^{131}I-labelled vaccinia virus in Gey's solution without bicarbonate. The rates of attachment to chick embryo cell monolayers are shown in Figs. 1 and 2. Very similar results were obtained with HeLa cell monolayers.

As expected from Brownian theory, a straight line is obtained when adsorbed virus is plotted against the square root of time. For both viruses the rates of adsorption by cell monolayers are very nearly one half of the maximum theoretical rate. The addition of calf serum in concentrations up to 5 % by volume made no significant difference to this result.

At 4° the rate of adsorption was reduced to 64 % of that at 20°. This is in agreement with the factor of 57 % expected from the increased viscosity of the medium at 4°, and implies that the adsorption of virus by cells is not dependent on temperature.

The adsorption of infective virus was followed by using unlabelled virus in higher dilution and recording as a function of adsorption time the number of plaques produced by fowl plague virus in chick embryo cell monolayers with the standard bicarbonate-agar overlay[3] and vaccinia virus with tris(hydroxymethyl)aminomethane-buffered agar overlay[4]. Representative results are given in Fig. 3 and Table I. Again the number of plaques increases in proportion to the square root of the adsorption time.

The validity of equation (2) was also tested by varying the volume of the fluid in the inoculum, which affects the term d (depth of fluid containing virus above

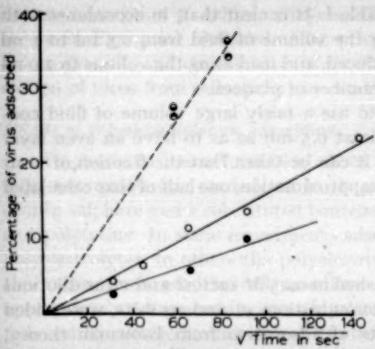

Fig. 1.

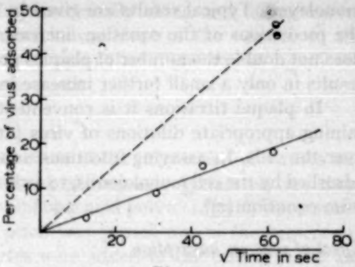

Fig. 2.

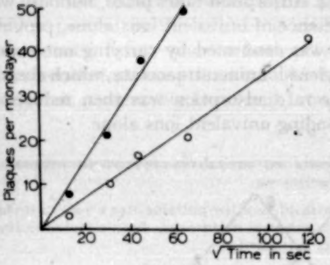

Fig. 3.

Fig. 1. Adsorption of labelled vaccinia virus by cell monolayers in 6-cm diameter Petri dishes. Interrupted line: percentage of virus colliding with monolayer at 20° calculated from Brownian theory. The percentages of virus adsorbed by glass (◒) and aluminium (⊖) surfaces, and by chick embryo cell monolayers (○) at 20° and (●) at 4° are shown.

Fig. 2. Adsorption of labelled fowl plague virus by chick embryo cell monolayers ○ glass ◒ and aluminium ⊖ surfaces, compared with the maximum calculated from Brownian theory (interrupted line).

Fig. 3. Increase in the number of vaccinia ○ and fowl plague ● plaques in chick embryo cell monolayers as a function of the square root of the time of adsorption.

TABLE I

EFFECTS OF INOCULUM VOLUME AND ADSORPTION TIME ON FOWL PLAGUE VIRUS PLAQUE PRODUCTION IN CHICK EMBRYO CELL MONOLAYERS (MEAN OF 4 DISHES)

Inoculum volume	*Time of adsorption (min)*	*Mean plaque count*
0.5 ml	15	13.5
	30	17.2
	60	29.0
	120	31.8
1.0 ml	15	18.2
	30	26.0
	60	37.5
	120	41.0
2.0 ml	15	19.8
	30	27.0
	60	36.5
	120	43.2

monolayer). Typical results are given in Table I. It is clear that, in accordance with the predictions of the equation, increasing the volume of fluid from 0.5 ml to 1 ml does not double the number of plaques produced, and increasing the volume to 2.0 ml results in only a small further increase in number of plaques.

In plaque titrations it is convenient to use a fairly large volume of fluid containing appropriate dilutions of virus (at least 0.5 ml) so as to have an even layer over the cells. In assaying infectious units it can be taken that the fraction of virus adsorbed by the cell monolayer is, to a close approximation, one half of that calculated from equation (2)[4].

Effect of ions on adsorption

Chick embryo cell monolayers were washed in 0.25 *M* sucrose and virus dilutions in sucrose, together with the appropriate concentrations of various salts, were added for 1 h. In calculating the maximum rate of adsorption from Brownian theory, a correction was introduced for the change in viscosity of the medium produced by sucrose.

The main results are summarized in Fig. 4. Adsorption takes place, not only with divalent or trivalent ions, but also in the presence of univalent ions alone, provided their concentration is sufficiently high. This was confirmed by carrying out experiments in the presence of 0.01 *M* sodium ethylenediaminetetraacetate, which chelates the multivalent ions in the media used. The rate adsorption was then reduced to that expected in the presence of the corresponding univalent ions alone.

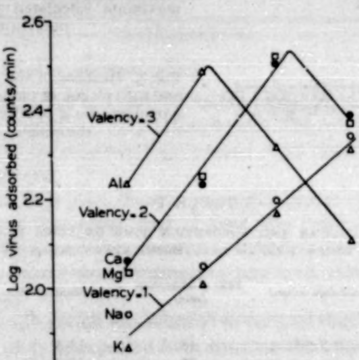

Fig. 4. Effect of cations on adsorption of labelled fowl plague virus by chick embryo cell monolayers. Count expected from Brownian Theory is 2·82.

As with adsorption by non-biological surfaces[1], straight lines are obtained when the logarithm of the adsorbed virus is plotted against the logarithm of the concentration of ions, the slope being much steeper for divalent ions than for univalent, and slightly steeper again for trivalent ions. At high concentrations, however, in contrast to the findings with non-biological surfaces, divalent and trivalent ions depress adsorption to cell monolayers. The symmetrically rising and falling curves shown in Fig. 4 are obtained. Adsorption was independent of the valency of the anions present, and control experiments showed again that there was no significant elution of virus from cells under the conditions used.

Effects of polyelectrolytes on adsorption

To obtain further information about the electrostatic interaction of viruses and cells, adsorption was carried out in the presence of various polyelectrolytes: chondroitin sulphate and a substituted benzene sulphonic acid polymer (53 K)[5], protamine and polylysine. In some experiments adsorption was carried out in the presence of polyelectrolytes, in others the polyelectrolytes were added to the cells or virus and the excess removed. The cell monolayers and virus were then washed once with appropriate medium lacking the polyelectrolyte (virus by high speed centrifugation followed by ultrasonic dispersion). It could be shown that both cells and virus take up ^{14}C-labelled 53 K in the presence of Ca^{++} and Al^{+++} ions used in representative experiments.

The main results are summarized in Table II. It is evident that both polyanions, whether present in the adsorbing medium or added to cells or virus beforehand, suppress adsorption, whereas both polycations in low concentrations enhance it. In higher concentrations protamine added to the virus, but not to the cells, depressed adsorption, presumably because it brought about clumping of the virus.

TABLE II

EFFECT OF POLYELECTROLYTES ON ADSORPTION OF ^{32}P-LABELLED FOWL PLAGUE VIRUS BY CHICK EMBRYO CELLS

Control in Gey's salt solution without bicarbonate. Polyelectrolyte concentration 1 mg/ml Gey's solution unless stated. Adsorption is expressed as a percentage of the maximum calculated from Brownian theory (equation 2).

Polyelectrolyte	*Where added*	*Adsorption*
Control	—	48
Protamine	Fluid	76
Protamine	Cells	82
Protamine	Virus	68
Protamine 10 mg/ml	Cells	72
Protamine 10 mg/ml	Virus	28
Polylysine	Fluid	68
Polylysine	Cells	76
Polylysine	Virus	70
Hyaluronic acid	Fluid	27
Hyaluronic acid	Cells	31
Hyaluronic acid	Virus	22
53K	Fluid	22
(substituted benzene	Cells	23
sulphonic acid polymer)	Virus	14

In Fig. 5 the effects of trivalent ions on adsorption in the presence of polyanions and polycations are shown. In the control, as before, low concentrations of Al^{+++} ions promote adsorption, whereas high concentrations depress it. In the presence of polylysine, intermediate concentrations of Al^{+++} ions increased adsorption virtually to the maximum rate calculated from Brownian theory, but high concentrations again depressed adsorption. In the presence of 53K adsorption was low throughout, but was not depressed by high concentrations of Al^{+++} ions.

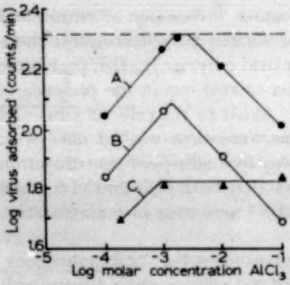

Fig. 5. Effect of concentration of Al^{+++} ions on adsorption of labelled fowl plague virus on chick embryo cells untreated (B), treated with polylysine (A) and with substituted benzene sulphonic acid polymer (C). The interrupted line shows the count expected from Brownian theory.

Effects of other reagents on adsorption

The effects of some other reagents on adsorption were investigated (Table III). Agents reacting with sulphydryl groups of cells and virus, including Hg^{++}, *p*-chloromercuribenzoate, iodoacetamide, N-ethylmaleimide and ω-bromacetophenone, produced no detectable change in the rate of attachment of virus to cells, although all these agents markedly depress virus infectivity[4].

TABLE III

EFFECTS OF VARIOUS REAGENTS ON ADSORPTION OF ^{32}P-LABELLED FOWL PLAGUE VIRUS BY CELL MONOLAYERS

Reagent	*Adsorption*
Control	54
p-chloromercuribenzoate	51
Hg^{++}	58
N-ethylmaleimide	49
Iodoacetamide	51
ω-bromocetophenone	53
Heat	50
Formalin 0.1 %	48
Acetic anhydride	14
$(UO_2)^{++}$	76
Trimethylenediamine	74

Heating the virus at 68° for 0.5 h, which abolishes infectivity, does not affect the rate of attachment to cells. Low concentrations of formalin (0.1 % or less for 4 h at 37°) had no detectable effect on adsorption, but higher concentrations of formalin (1 %) for 4 h depressed adsorption. Treatment of the virus with 10^{-3} *M* acetic anhydride at pH 7.8 in bicarbonate buffer, followed by neutralization to pH 7.0, reduced the rate of adsorption quite considerably.

On the other hand, in the presence of uranyl ions in high concentration, adsorption was increased. The same was achieved by trimethylenediamine in high concentration. The depressed adsorption shown with divalent cations was not apparent with this compound, in which the two positively charged groups are separated by three carbon atoms.

Effect of pH on adsorption

Fowl plague virus labelled with ^{32}P was made up in various buffers covering the range pH 3.0 to 7.0. Experiments above pH 7.5 failed because the cell monolayer lifted from the glass. The adsorption of virus as a percentage of the maximum calculated from Brownian theory is shown in Fig. 6. It will be seen that adsorption is greatest at pH 5.5, irrespective of the nature of the buffer ions present. Between pH 5.5 and 7.0 there is a slight decline in the efficiency of adsorption, and between pH 5.5 and 4.0 there is a marked decline. Below a minimum at pH 4.0 the percentage of virus adsorbed rose again.

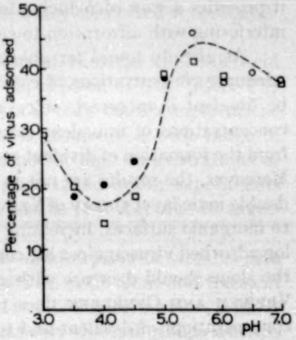

Fig. 6. Effect of pH on adsorption of fowl plague virus by chick embryo cell monolayers. Virus adsorbed is expressed as a percentage of the maximum value calculated from Brownian theory. Glycine acetate phosphate buffer ○, acetate buffer ●, phosphate buffer □.

DISCUSSION

Attempts have been made to use a rate constant of the type applicable to adsorption of phage by bacteria to describe the adsorption of animal viruses by cell monolayers:

$$f = 1 - \exp\{-Knt\}$$

where *f* is the fraction of virus adsorbed, *n* is the number of adsorbing cells, *t* is the time after commencing adsorption and *K* is the rate constant.

As previously pointed out[1], the use of such equations to define the rate of adsorption of virus by cell monolayers is not justified. Some measurements of adsorption of animal viruses by cell layers have already been published[7,8], but these have been based on infectivity determinations showing rather a wide scatter. Moreover, the conditions under which adsorption takes place have not been varied sufficiently to give useful information about the adsorption process.

In the case of phage[9-11] adsorption occurs efficiently in the presence of sufficient concentrations of cations. PUCK AND TOLMACH[12] concluded that the main interacting groups are the amino groups of phage and the carboxyl groups of host bacteria. DIRKX *et al.*[11] have had some measure of success in defining the conditions under which phage adsorption takes place in terms of the ionic double layer theory of VERWEY AND OVERBEEK[13].

From our experiments certain general conclusions can be drawn. First is a point of practical use in experiments involving plaque titrations and quantitative studies of virus growth. The rate of attachment of virus to cell monolayers is very close to half the collision frequency calculated from Brownian theory. Thus by halving the value for N obtained by using equation (1), a good estimate of adsorbed virus can be made. This appears to hold, not only for the viruses studied by us (vaccinia and fowl plague), but also for herpes simplex[7] and poliomyelitis[8], four viruses that probably differ appreciably in surface composition. Adsorption to non-biological surfaces, on the other hand, proceeds at a rate almost identical with the collision frequency[1]. Another difference is apparent when protein is added to the medium. No effect on adsorption of viruses by cells was detected, but adsorption by glass and other surfaces was strongly depressed. This again has practical application, for it provides a way of reducing loss of virus through adsorption to glassware without interfering with adsorption to cells.

As already found for phage, adsorption of animal viruses to host cells requires adequate concentrations of cations. However, the usual implication that these must be divalent is incorrect, since adsorption takes place in the presence of adequate concentrations of univalent cations alone. Thus the concept that adsorption results from the formation of divalent cationic bridges between viruses and cells is invalidated. Moreover, the results are not in agreement with the quantitative predictions of the double ionic layer theory of VERWEY AND OVERBEEK[13]. As in the adsorption of viruses to inorganic surfaces, increasing the valency of the cation increases the slope of the log adsorbed virus against log concentration of ion; whereas according to their theory the slope should decrease with cations of higher valency. In addition the theory of VERWEY AND OVERBEEK does not explain the suppression of adsorption by high concentrations of divalent and trivalent cations.

Third, some tentative conclusions can be drawn about the interacting surfaces. Although influenza virus (which is related to fowl plague) has about 11.5 % phospholipid[14], electrophoretic measurements show that it is isoelectric at about pH 5.3[15]. In other words the surface charge is dominated by amino and carboxyl groups of protein rather than strongly acidic groups of phospholipid. The animal cells that have been studied retain a negative charge at a much lower pH, and the electrophoretic mobility in the presence of uranyl and other ions suggests that the surface charge is dominated by strongly acidic, especially phosphate, groups[16]. At pH 5.5 to 7.6—the conditions under which most experiments are undertaken—cells and virus particles are both negatively charged. There is thus an electrostatic barrier to adsorption in the form of layers of positively charged ions concentrated around the surfaces of both cells and viruses. The thickness of this layer can be reduced by raising the concentration of cations in the suspending fluid. Above a critical concentration the virus can come sufficiently close to the cells for short range attractive forces to bind them together. The temperature coefficient, effect of ions and other properties make it clear that the primary attachment is electrostatic. The main interacting groups are likely to be amino groups of the virus and strongly acidic, mainly phosphate, groups of host cells. This is shown by mild acetylation of the amino groups of the virus, which depresses adsorption to host cells (Table III), although it makes no detectable difference to the attachment of virus particles to positively charged aluminium surfaces. Acetylation of proteins under these conditions does not denature them, but lowers the isoelectric point considerably[17]. The effect of uranyl ions in promoting adsorption is consistent with the well-known affinity of these ions for phosphoric acid groups[16, 18]. Agents reacting with sulphydryl groups of cells and viruses were without effect.

The increase in adsorption of virus between pH 7.0 and 5.5 can plausibly be explained by suppression of ionization of carboxyl-groups, reducing the net negative charge of the virus and enhancing adsorption. At about the isoelectric point of the virus there is considerable aggregation of virus particles to form large, slowly diffusing units, so that the rate of attachment to cells is much reduced.

As expected, polyanions which increase the net negative charge of both host cells and virus, or each separately, diminish adsorption. Low concentrations of polycations, on the other hand, increased adsorption. Higher concentrations added to cells increased adsorption, but added to virus particles they decreased it, probably owing to aggregation of virus particles.

The reduction in virus infectivity in the presence of benzene sulphonic acid reported by HEYMANN *et al.*[19] may be due simply to suppression of adsorption. The interaction with ribonuclease quoted by these authors is only one example of the reactivity of polyanions of this type towards the basic groups of a wide range of proteins[20]. Reduction of virus infectivity in the presence of high concentrations of lysozyme may be analogous to the suppressed adsorption noted in our experiments in the presence of high concentrations of protamine, and is probably due to aggregation of virus particles.

The most puzzling observation is the suppression of adsorption of virus to cells (but not to glass and other surfaces) by high concentrations of divalent and trivalent cations. The relevant difference between biological and non-biological surfaces is likely to be that the former possess both positively and negatively charged groups whereas the latter have only negative charges. An important feature is illustrated in Figs. 4 and 5. When the logarithm of the adsorbed virus is plotted against the logarithm of the concentration of cations, linear increases are obtained. The slopes depend upon the valency of the cations and the size of the particles adsorbed, and have the same values for adsorption on to cells and non-biological surfaces. At certain ionic concentrations, however, in the case of adsorption to cells, the rising curve abruptly changes to a falling one, the slope remaining unaltered. It is this sudden change of sign that prevents the curve from rising to give an adsorption rate equal to the collision frequency, as is found with non-biological surfaces. The inhibited adsorption in the presence of multivalent cations is not due to elution, which was excluded in some experiments. The symmetry of the rising and falling curves does not suggest the intervention of an entirely new factor.

Existing theoretical analyses of the interactions between dissimilar charged surfaces during adsorption are far from complete[21]. It is possible that firm cation binding by both surfaces, of the type analysed by STERN[22], plays a part in the depressed adsorption of viruses in high concentrations of multivalent ions. The suggestion[1] that it is the size of ions as much as their charge that determines their effectiveness in promoting adsorption is consistent with this interpretation.

ACKNOWLEDGEMENTS

We are indebted to Dr. H. G. PEREIRA for help in the preparation of radioactive fowl plague virus and some other experiments, to Drs. C. KAPLAN and A. S. McFARLANE for supplying and iodinating the vaccinia virus, to Dr. H. J. ROGERS for donating polylysine and substituted benzene sulphonic acid polymer (including a ^{14}C-labelled sample) and to Mrs. P. SMITH for technical assistance.

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LIGHT-SCATTERING STUDIES ON ASCITES TUMOR CELL RNA

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(Received October 3rd, 1959)

SUMMARY

The molecular parameters of the high molecular weight, ribonucleic acid, prepared from Ehrlich ascites tumor cells have been determined. This RNA appears to consist of two main components of molecular weights of $2.3 \cdot 10^6$ and $3.2 \cdot 10^5$. Under conditions close to physiological, these molecules can be described best as compact rods 40–45 Å in thickness.

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Biochim. Biophys. Acta, 40 (1960) 410–417

INTRODUCTION

The discovery of GIERER AND SCHRAMM[1] that TMV ribonucleic acid (RNA) carries the viral infectivity has precipitated a number of studies on the molecular state of this material. As a result, GIERER has shown that the molecular weight of the infective RNA is close to two million[2], and that TMV-RNA is a monodisperse substance. Further, GIERER has found that with a change in ionic strength of the medium this RNA can undergo a reversible transition from a compact to a loosely coiled structure[2].

Since the preparation by COLTER AND BROWN[3] of high molecular weight RNA from the cytoplasmic fraction of Ehrlich ascites tumor cells, it became of interest to characterize these molecules which are mammalian in origin. In preliminary reports[3,4], it has been shown that the high molecular weight fraction of this RNA is paucidisperse. It is the purpose of this paper to present a detailed description of studies on the physical-chemical properties of these RNA molecules in solution.

EXPERIMENTAL

The RNA was prepared by the phenol extraction technique according to the method of COLTER AND BROWN[3]. Following the phenol extraction, the high molecular weight RNA was precipitated in each case with 1 N NaCl. Sample I was lyophilized, sample II was frozen and stored at —40°, being thawed only a few hours before use. Before light scattering measurements these samples were dissolved in 0.1 ionic strength buffer (0.02 Na phosphate, 0.08 NaCl) of pH 6.8 (sample I) and 7.0 (sample II), dialyzed overnight against it in the cold, and the dialyzate was used as diluent in the light scattering measurements, carried out at 25°. In the case of sample III, the pellet obtained on centrifugation of the salt precipitate was immediately resuspended in a cold pH 7.0 buffer (ionic strength: 0.01 Na phosphate, 0.14 NaCl), dialyzed for 2 h against the same buffer at 6° with stirring, and promptly used for the light scattering measurements at 10–12°.

The light scattering measurements on samples I and II were carried out at 25° in a 20-ml WITNAUER type[5] cell on the BRICE photometer[6] with 2 mm slit optics between 25° and 135°. The solutions were clarified by preliminary centrifugation in a Spinco Model L* centrifuge followed by filtration through an ultra-fine sintered glass filter of special design[7,8]. Dilutions were carried out using a modified DINTZIS technique[9]. Successive concentrations were measured in a single cell, starting with pure solvent, using a microburette for addition of a concentrated stock solution and mixing with a magnetic stirrer. In the case of sample III, all manipulations and measurements were made at 10–12° unless otherwise stated. Solutions were prepared by quantitative dilution of the stock solution. Light scattering measurements were made between the angles of 19° and 140° with 1 mm entrance slits in the photometer, using a recently designed cell[10]. The latter, which consists of a cylindrical cell[5] fused to an ultrafine filter, permits rapid angular measurements on as little as 8 ml of solution without the necessity of transferring solution from filter to cell. By using a separate cell-filter for each solution it was possible to clarify six individual solutions and to measure their angular envelopes in less than 2.5 h.

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Concentrations were measured by u.v. absorption at 260 mμ using the absorbtivity[3] of 21.0 l g^{-1} cm^{-1}. The value of dn/dc used was 0.1716[4]. Sedimentation distribution curves were derived from u.v. absorption data obtained with the Spinco Model E ultracentrifuge.

RESULTS AND DISCUSSION

Molecular weight and size of RNA

The light scattering data were plotted in all cases in ZIMM[11] and YANG[12] plots and showed a weak angular dependence. The presence of some polydispersity was attested to by the slight curvature of the zero concentration envelopes. These results are presented in Figs. 1, 2 and 3 for Samples I, II and III, respectively. The radius of gyration, the molecular weight obtained and the partial specific volume of RNA (0.528)[13] are inconsistent with a spherical model. The rod model, found by BROWN in electron microscopic studies[14] is in reasonable agreement with the data. In the data analysis, multi-component effects[9] were neglected. Molecular parameters calculated from the light scattering data for the rod-shaped molecules are presented in the Table. The values of the weight average molecular weight ($\overline{M_w}$) were obtained from the extrapolation of the ZIMM plot to zero angle and zero concentration, the slope of the YANG plot at zero concentration, and the extrapolation to zero concentration of the 90° data, corrected for intrinsic dissymmetry (z) at 45 to 135°. In each case, all three values are found to be in good agreement. The weight average length, $\overline{L_w}$, was obtained from the intrinsic dissymmetry, and the Z-average length, $\overline{L_z}$ from the limiting slope of the zero concentration curve of the ZIMM plot and the intercept of the YANG plot.

Since it has been reported that heavy metals[15] have a strong influence on the shape of the RNA molecule, a comparison was made of light scattering from solutions containing 0.01 % Versene with that from Versene-free solutions. The results shown in Fig. 3 indicate that any trace metals that might be present have no discernible effect on the scattered intensity and hence on $\overline{M_w}$ or the size of RNA.

BROWN[14] has shown in electron microscope studies on similar preparations of

TABLE I

MOLECULAR PARAMETERS OF EHRLICH ASCITES TUMOR CELL RNA

	Sample I	*Sample II*	*Sample III*	*Sample III (calculated)**
$\overline{M_w}$, ZIMM	$1.40 \cdot 10^6$	$1.18 \cdot 10^6$	$1.49 \cdot 10^6$	$1.6 \cdot 10^6$
$\overline{M_w}$, YANG	$1.32 \cdot 10^6$	$1.17 \cdot 10^6$	$1.48 \cdot 10^6$	
$\overline{M_w}$, Dissym	$1.34 \cdot 10^6$	$1.09 \cdot 10^6$	$1.37 \cdot 10^6$	
Z (Dissym)	1.24	1.14	1.20	—
$\overline{L_w}$	890 Å	690 Å	810 Å	920 Å
Lim slope (ZIMM)	$6.2 \cdot 10^{-7}$	$4.2 \cdot 10^{-7}$	$(4.0–7.5) \cdot 10^{-7}$	—
Lim slope (YANG)	$4.1 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$	$7.5 \cdot 10^{-7}$	—
ϱg^2	$(9.71–17.6) \cdot 10^4$ Å^2	$(7.10–10.3) \cdot 10^4$ Å^2	$(11.2–22.4) \cdot 10^4$ Å^2	—
$\overline{L_z}$	1,060–1,500 Å	925–1,110 Å	1,160–1,640 Å	1,310 Å
$\overline{M}_z$, calc.	$(2.14 \pm 0.36) \cdot 10^6$	$(1.69 \pm 0.15) \cdot 10^6$	$(2.34 \pm 0.42) \cdot 10^6$	$2.18 \cdot 10^6$

* Calculated from bimodal distribution of 63 % of 34-S and 37 % of 18-S component.

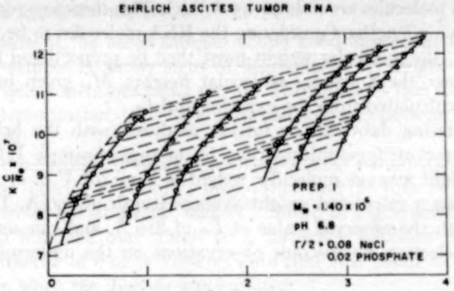

Fig. 1. Light scattering data of preparation I RNA (Zimm plot).

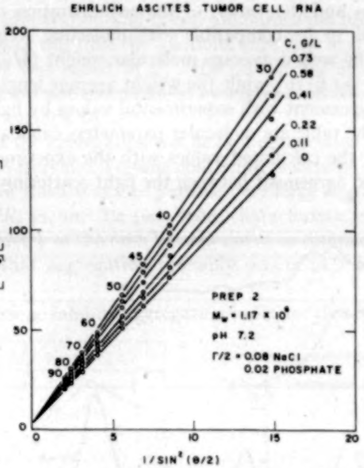

Fig. 2. Light scattering data of preparation II RNA (Yang plot).

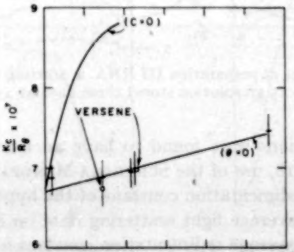

Fig. 3. Light scattering envelopes of preparation III RNA in the presence and absence of versene.

RNA that the RNA molecules are rods 40–45 Å thick. This thickness remains constant for particles of varying lengths. Considering the RNA molecules to be cylinders with a diameter of 43 Å, the molecular weight must then be proportional to the particle length. In this manner, the Z-average molecular weights, $\overline{M}_z$, given in the last line of the Table, were calculated from the experimental $\overline{L}_z$.

The light scattering data can be further analyzed with the help of electron microscopic and ultracentrifugal information. In the case of sample III, a cylindrical molecule with a weight average molecular weight of $1.49 \cdot 10^6$, $\overline{V}$ of 0.52_8 and a base diameter of 43 Å has a calculated weight average length of 890 Å. This is in very good agreement with the observed value of $\overline{L_w}$ of 810 Å, and can serve as further verification of the electron microscopic observations on the uniformity of particle thickness.

Ultracentrifugally, sample III is found to contain essentially two components: 63 % of a 34-S component and 37 % of a 18-S component, as shown in Fig. 4. The shape of the molecules and the values of the sedimentation constants of the two components are found to be compatible with molecular weights of $2.3 \cdot 10^6$ (63 %) and $3.2 \cdot 10^5$ (37 %)[14]. The weight average molecular weight ($\overline{M_w}$) calculated for such a bimodal distribution is $1.6 \cdot 10^6$, while the weight average length, $\overline{L_w}$, is 920 Å, both of which are in good agreement with experimental values by light scattering. Shown in the last column of the table are molecular parameters calculated for such a distribution. Comparison of the calculated values with the experimental data, shown in column 4, point to good agreement between the light scattering and ultracentrifugal data.

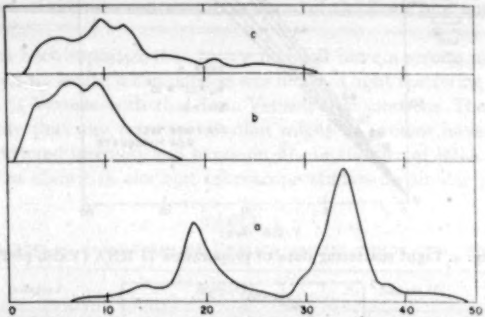

Fig. 4. Ultracentrifugal diagrams of preparation III RNA. a, starting material; b, 2.9 g/l solution stored three days at 25°; c, 0.25 g/l solution stored three days at 25° in presence of versene.

Similar RNA preparations were found to have an intrinsic viscosity of 0.40[3]. Assuming an axial ratio of 18, use of the SCHERAGA-MANDELKERN equation[16] results in a value of 34-S for the sedimentation constant of the hypothetical RNA molecule represented by the weight average light scattering data on Sample III. The experimental value of the weight average sedimentation constant is 31-S. This can be compared further with the weight average sedimentation constant of 27-S calculated according to the method of GIERER[2] for $\overline{M_w} = 1.5 \cdot 10^6$.

Degradation and aggregation of RNA

A comparison of the three samples shows good agreement between samples I and III, while sample II shows signs of degradation, its weight average molecular weight and particle dimensions being smaller. This indicates that freezing and thawing results in depolymerization, while lyophilization seems to have no pronounced effects on the molecules.

In order to determine if significant changes occur in the state of aggregation of RNA during the interval between its preparation and the time of measurement, time studies were carried out at 10–12° and 25°. Shown in Fig. 5 are representative light scattering data obtained over about 68 h for RNA solutions (sample III) in the presence and absence of 0.01 % Versene. Zero time was taken to be the time during the preparation when the dialysis was initiated.

As Fig. 5 illustrates even at 10–12° there is a slow degradation of RNA to the extent of a change in $\overline{M_w}$ of about 10 % in 26 h. Changes in $\overline{M_w}$ occurring during the interval between preparation and initial measurements are thus minor, particularly since during 40 % of this time the solutions were kept at 6°. As Fig. 5 illustrates, when the temperature was raised to 25°, the rate of degradation increased but in Versene-free solutions was not accompanied by aggregation. This is most evident from the data taken at an angle of 19°.

The magnitude of the aggregation can be best appreciated by comparing angular envelopes for Versene-free and Versene-containing solutions. Such plots, shown in Fig. 6, indicate that in the absence of Versene very large aggregates are formed, the maximum in the Kc/R_θ *vs.* $\sin^2 \theta/2$ plot being characteristic of very large spherical particles[17]. The asymmetry of the 68-h Versene curve as compared to the 8-h Versene-free curve indicates that aggregation probably occurs to some extent even in the presence of Versene.

The fact that Versene inhibits aggregation indicates that in spite of the fact that

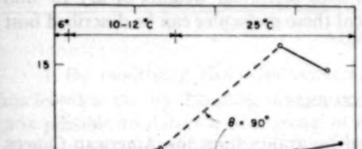

Fig. 5. Changes in light scattering of preparation III RNA solutions (0.25 g/l) during storage at various temperatures. ○—○, no Versene; ●—●, in the presence of 0.01 % Versene.

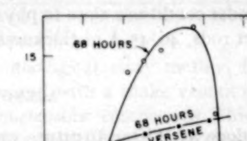

Fig. 6. Scattering envelopes of preparation III RNA solutions (0.25 g/l) after various durations of storage. ○—○, no Versene; ●—●, in the presence of 0.01 % Versene.

RNA was prepared with redistilled phenol the final solutions contain some metal ions. Nonetheless, as Fig. 3 illustrates these metal ions have no effect upon the scattering of light from RNA solutions at high ionic strength and thus could have had no marked effect on either the molecular weight or size. These results contrast with the findings of Haschenmeyer, Singer and Fraenkel-Conrat[15], who showed that TMV-RNA prepared in the presence of metals such as Ca^{2+} had a more compact configuration in solutions of low ionic strength than that of metal-free preparations. It would appear that this is simply a polyelectrolyte effect, *i.e.*, at low ionic strength the bound metal ions reduce the net charge and the electrostatic repulsion, thereby decreasing the average molecular end to end distance.

While the light scattering data emphasize the aggregative processes, and in the case of the Versene-containing RNA sample, point to an apparent absence of gross changes, sedimentation measurements indicate the contrary. Shown in Fig. 4 are sedimentation distribution curves of samples kept for three days at 25°. Extensive degradation has occurred at both high (2.9 g/l) and low (0.25 g/l) RNA concentrations. In the case of the 0.25 g/l samples, when Versene was absent, degradation proceeded to a point where no sedimentable material could be observed. Similar degradative changes of RNA have been observed by Gavrilova *et al.*[18] and by Littauer and Eisenberg[19], the latter authors having demonstrated that metal ions can promote RNA degradation.

The above data show that while RNA is capable of aggregating under the influence of metal ions, the more important process is a degradation, also apparently promoted by metal ions.

CONCLUSION

The high molecular weight fraction of cytoplasmic Ehrlich ascites tumor cell RNA appears to consist of two main components with molecular weights of $2.3 \cdot 10^6$ and $3.2 \cdot 10^5$. Under conditions close to physiological these molecules can be described best by compact rods, 40–45 Å in thickness.

ACKNOWLEDGEMENTS

The work done at Wistar Institute was aided by grants from the American Cancer Society (E-88, E-89) and from the Samuel E. Fells Fund.

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STUDIES ON DEOXYNUCLEOSIDIC COMPOUNDS

I. A MODIFIED MICROBIOASSAY METHOD AND ITS APPLICATION TO SEA URCHIN EGGS AND SEVERAL OTHER MATERIALS*

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(Received August 10th, 1959)

SUMMARY

1. By modifying Hoff-Jørgensen's microbiological assay method for deoxynucleosides, *viz.* by digesting samples to be assayed with a snake venom enzyme, it was possible to detect a new group of deoxynucleosidic compounds. This group of deoxynucleosidic compounds, differing from either simple deoxynucleosides or deoxynucleotides, were tentatively designated as "masked" deoxynucleosidic compounds.

2. "Masked" deoxynucleosidic compounds were detected in various tissues such as sea urchin eggs and embryos and mammalian tissues including tumors.

3. The relative amounts of "masked" deoxynucleosidic compounds compared with DNA or simple deoxynucleosidic compounds in eggs and early embryos were markedly higher than those in adult tissues. In this connection, the biological significance of "masked" deoxynucleosidic compounds was discussed.

Abbreviations: DNA, deoxyribonucleic acid; PCA, perchloric acid.

* Supported in part by a grant from the Rockefeller Foundation and a subsidy from the Tokai Gakujutsushoreikai.

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INTRODUCTION

It is generally assumed that DNA carries the genetic information of the living systems, and the physical and chemical properties as well as the biological role of this macromolecule have been studied extensively. However, relatively little is known about the natural occurrence of acid-soluble deoxynucleosidic compounds, which may play some role in the active metabolism of DNA and cell proliferation.

To perform an experiment on the nature and role of these deoxynucleosidic compounds, it seems desirable to select a biological system in which rapid multiplication and active DNA metabolism take place. Further, an analytical method is required by which a minute amount of deoxynucleosidic compounds can be specifically determined in the presence of a relatively large amount of ribonucleosidic compounds.

In this study, unfertilized eggs and early embryos of sea urchin (*Hemicentrotus pulcherrimus*) were used as the main experimental material. The unfertilized eggs are thought to be in the particular physiological condition preceding the rapid cell division (cleavage) that ensues soon after fertilization[1]. On the other hand, embryos of early developmental stages are regarded as representative of the system in which rapid cell multiplication is under way.

As an analytical method, we employed the microbiological method, originally devised by HOFF-JØRGENSEN[2,3] for the analysis of deoxynucleosides and deoxynucleotides; it has been found very useful because of its high specificity and sensitivity.

During the progress of this investigation, an interesting fact came to our notice. If pretreatment by snake venom digestion were carried out prior to the original microbiological assay, hitherto undescribed deoxynucleosidic compounds were found to be detectable in acid-soluble extracts of eggs and embryos of sea urchin, toad embryos, several animal tissues including tumors, and microorganisms.

In this paper, details of our modified microbiological procedure and several results obtained by applying this procedure to eggs and embryos of the sea urchin and tissues of the rat and mouse are described.

A preliminary report has appeared elsewhere[4].

MATERIALS AND METHODS

Eggs and embryos of the sea urchin. Samples of mature unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*, were prepared as follows. The spawning was induced by introducing an isotonic KCl solution into the body cavity. After filtration through gauze, the eggs were exposed to HCl–sea water (pH 4.5–5.5) to remove the jelly coat, and washed several times with filtered sea water. The eggs were further washed with isotonic KCl, frozen with dry ice–ethanol and lyophilized. Only eggs from the lots showing fertilization reaction upon insemination were used for the experiment.

To obtain embryos of a given developmental stage, the jelly-less eggs were inseminated with sperm suspension prepared by diluting the "dry sperm" ten thousand-fold with sea water, and cultured at room temperature after washing several times with isotonic KCl and sea water to remove excess spermatozoa. Care was taken to avoid crowding of the eggs or embryos during culture. The embryos that reached the desired stage of development were collected by centrifugation, washed with isotonic KCl, frozen and lyophilized.

The lyophilized samples of eggs and embryos were defatted with ethanol–ether (1:3) to facilitate the subsequent extraction with PCA. The defatting procedure did not remove any appreciable amount of deoxynucleosidic compounds.

Rat tissues and tumors. Tissues of the rat and mouse were obtained from decapitated animals as quickly as possible and immediately homogenized with cold 0.2 *N* PCA or frozen with dry ice–ethanol. The frozen tissue could be stored at —20° for a few days. In the case of ascites tumors, the animals were killed by cervical fracture and the ascitic fluid was drained through an abdominal incision into tubes surrounded by ice. The cells were sedimented by centrifugation at 1,000 × *g* for 7 min. The packed cells were extracted with cold 0.2 *N* PCA. 1 *N* PCA was added to the supernatant fluid to give a final concentration of 0.2 *N*. Tumors* used in this experiment were ascites hepatoma 7974[5] (rat), sarcoma 180 (mouse), and Ehrlich tumor (mouse).

Thymidine and other chemicals were obtained commercially.

DNA was prepared from bovine spleen by the detergent method[6]. The fibrous product containing 8.5 % of phosphorus was contaminated by less than 2 % of RNA.

Enzymic casein hydrolysate. This was prepared by digestion of casein (Nutritional Biochemicals) with papain. An amount of concentrated hydrolysate containing 76 mg nitrogen (equivalent to 500 mg protein) was added to each 100 ml of the basal medium.

Snake venom. Vacuum-dried powder of venom of *Trimeresurus flavovidridus* or *Agistrodon blomhoffi* was used. Sometimes the recovery in microbioassay of DNA was found incomplete owing to the sample of venom used. Therefore such samples of venom as gave satisfactory recovery of DNA were selected for subsequent use.

Microbioassay. Deoxynucleosidic compounds were assayed microbiologically with *Lactobacillus acidophilus* R-26. The method was essentially the same as that described by HOFF-JØRGENSEN[2,3] except for the following modifications: uracil (2 mg/ml) and pyridoxine (100 μg/ml) were added to purine-pyrimidine and vitamin solutions respectively, and the concentrations of DL-tryptophan, L-cysteine and thioglycolic acid were doubled.

The overall procedure for the determination of deoxynucleosidic compounds, with or without snake venom digestion, is described in RESULTS AND DISCUSSION.

Determination of DNA. DNA contents of eggs and embryos were determined microbiologically, and those in rat tissues chemically[7].

RESULTS AND DISCUSSION

Effect of snake venom digestion

First, the acid-soluble extracts of eggs and embryos were analysed for their deoxynucleosidic compounds by the original method of HOFF-JØRGENSEN. As long as the original procedure was used, deoxynucleosidic material was scarcely detectable in these extracts.

By chance, it was found that digestion of the extracts with snake venom enzyme before microbioassay made the extracts highly active in supporting the growth of the test organism (Fig. 1).

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* Tumor samples were kindly provided by Dr. K. KAZIWARA of Research Laboratories, Takeda Pharmaceutical Industries, Ltd.

There are three possible ways of interpreting this activating effect of the venom digestion. Firstly, the venom might activate a trace amount of DNA present in the acid-soluble extracts. But this was excluded by the following facts, indicating the absence of any detectable amount of DNA in the extracts: pancreatic deoxyribonuclease, which is known to activate DNA microbiologically[2,3], could not replace the venom in activating the extracts, and the active principle of the extracts was found to be completely dialyzable through cellophane membrane, whereas DNA is non-dialyzable.

The second possibility, that the venom digestion might remove an inhibitor, is highly unlikely because the addition of the acid-soluble extracts to a thymidine solution did not inhibit the recovery of the latter in the microbioassay. If there were any inhibitor, the recovery of thymidine should be depressed.

Thus we were led to accept the third possibility that there exists a new group of deoxynucleosidic compounds which are, in fact, activated by venom treatment and therefore not detectable by the usual microbiological method.

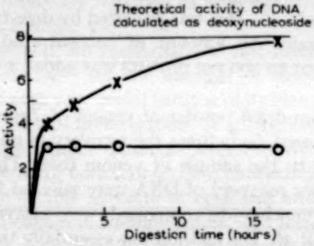

Fig. 1. Activation of acid-soluble deoxynucleosidic compounds of sea urchin eggs (O—O) and DNA (×—×) by snake venom digestion. Activity expressed as $m\mu$mole thymidine/ml digestion mixture. Conditions for digestion: snake venom, 250 μg/ml; $MgSO_4$, 0.01 *M*; potassium maleate, 0.06 *M*, pH 6.8; incubated at 37°.

We tentatively designated this group of deoxynucleosidic compounds as "masked" deoxynucleosidic compounds[4]. It is evident that the "masked" deoxynucleosidic compounds are neither simple deoxynucleosides, deoxynucleotides, nor products obtained by degradation of DNA with deoxyribonuclease, because the latter compounds are all active toward *L. acidophilus* without any pretreatment.

Analytical procedure with special reference to "masked" deoxynucleosidic compounds

Before attempting to clarify the chemical nature and biological significance of "masked" deoxynucleosidic compounds, it seemed necessary to establish a routine analytical procedure.

Tissues (dry defatted powder or fresh tissues weighing 10–100 mg or 50–500 mg, respectively) were extracted three times or more with 2–4 ml portions of cold 0.2 *N* PCA, using a glass homogenizer. The combined extracts were neutralized with 2 *N* KOH, kept overnight in a refrigerator and centrifuged to remove potassium perchlorate. The supernatant was diluted or concentrated to a given volume (usually 10 ml).

An aliquot of less than 4.0 ml was taken from this neutralized extract. To it was added 1.0 ml of maleate–magnesium sulphate–venom solution of pH 6.8, containing 0.3 mmole potassium maleate, 0.05 mmole magnesium sulfate, and 2.0 mg snake venom, and the mixture was diluted to 5.0 ml with water and incubated for 3–16 h at 37° (under a layer of toluene or hexane). The digesting reaction was stopped by immersing a glass tube containing the reaction mixture in a boiling water bath for 5 minutes. The venom-treated acid-soluble fraction so obtained was referred to as ASt.

Another aliquot from the neutralized PCA extracts was treated in the same way except that snake venom was omitted from the reaction mixture and the resulting solution was referred to as ASu.

The residue from the cold PCA extraction was washed twice with cold ethanol and once with ether, dried, incubated with 2–5 ml 0.5 *N* KOH overnight at 37°, neutralized with 0.5 *N* maleic acid, and diluted to a given volume. The rest of the treatment of this acid-insoluble fraction was the same as that of acid-soluble fraction, and two separate aliquots served for making two samples, AIt and AIu, which were incubated with and without snake venom, respectively.

The four samples, ASu, ASt, AIu, and AIt, thus obtained, were assayed for their deoxynucleosidic growth activity according to HOFF-JØRGENSEN. Thymidine was used as an assay standard and all results were expressed as mμmoles thymidine equivalent.

As is evident from the above procedure, ASu represents the amount of simple deoxynucleosides and deoxynucleotides, and the difference, ASt minus ASu, represents that of "masked" deoxynucleosidic compounds. The difference, AIt minus AIu, on the other hand, represents the amount of DNA. In our experience, AIu was always microbiologically inactive, so that the analysis of AIu could usually be omitted.

Amount of simple and "masked" deoxynucleosidic compounds in eggs and embryos of sea urchin and tissues of the rat and mouse

Eggs and embryos of the sea urchin, the liver, kidney, spleen and tumors of the rat, and tumors of the mouse were analysed for their deoxynucleosidic compounds by our modified procedure.

Analytical results in sea urchin eggs and embryos at various developmental stages are summarized in Table I and those in rat and mouse tissues in Tables II and III.

Several interesting points are seen from these tables. First, as already mentioned, in eggs and embryos almost all of the acid-soluble deoxynucleosidic compounds are in the "masked" form and scarcely any simple deoxynucleosides or deoxynucleotides are found. This is in sharp contrast with the situation in adult rat tissues, where a relatively large portion of the acid-soluble deoxynucleosidic compounds consists of simple deoxynucleosides and/or deoxynucleotides. Secondly, the amount of "masked" deoxynucleosidic compounds remains almost constant during the early cleavage but shows a marked decrease during the later cleavage stage, when the rate of DNA synthesis and cell division gradually decreases.

Finally, it will be seen that the ratio of the acid-soluble "masked" deoxynucleosidic compounds to DNA is the highest in eggs before or immediately after fertilization and becomes lower with the progress of development, whereas the ratio is very low in the adult mammalian tissues.

All these facts suggest that the "masked" deoxynucleosidic compounds participate actively in DNA synthesis or some other biosynthetic reactions taking place in

TABLE I

DEOXYNUCLEOSIDIC COMPOUNDS CONTENT OF SEA URCHIN EGGS AND EMBRYOS

Material	*ASu**	*ASt**	*"Masked"* deoxynucleosidic compounds (ASt–ASu)*	*DNA* (AIt)*	*"Masked"*** deoxynucleosidic compounds / DNA*
Unfertilized eggs:					
No. 1	4.0	75	71	14	5.1
2	6.1	69	63	40	1.6
3	8.3	112	104	46	2.3
4	5.0	87	82	35	2.3
5	5.0	86	81	34	2.4
6	5.0	80	75	30	2.5
Embryos:					
stage 1 cell	—**	78	78	12	6.5
1 cell	—**	106	106	16	6.6
1 cell	—**	134	134	17	7.9
2 cells	—**	104	104	29	3.6
4 cells	—**	103	103	43	2.4
8 cells	—**	117	117	111	1.1
8 cells	—**	126	126	108	1.2
16 cells	—**	116	116	140	0.82
early blastula	2.0	46	44	1,350	0.033
early blastula	1.0	35	34	1,320	0.026
hatching blastula	—**	18	18	1,660	0.011

* Expressed as mμmole thymidine equivalent/100 mg dry weight. Since no appreciable change of dry weight per egg or embryo is observed during the early developmental stage studied, the values calculated on the basis of dry weight are roughly proportional to the amounts per egg or embryo.

** Trace.

*** The ratio of values of column 4 to those of column 5.

TABLE II

DEOXYNUCLEOSIDIC COMPOUNDS CONTENT OF RAT TISSUES

Material		*ASu**	*ASt**	*"Masked" deoxynucleosidic compounds (ASt–ASu)**	*DNA***	*"Masked"*** deoxynucleosidic compounds / DNA*
Liver:	No. 1	42.7	92.3	49.6	8,710	0.0057
	2	35.7	62.4	26.7	8,560	0.0031
	3	25.9	51.7	25.7	8,880	0.0028
	4	26.6	90.0	64.1	7,800	0.0082
	5	37.8	68.4	30.6	8,070	0.0038
Spleen:	No. 1	93.1	156	42.9	37,200	0.0012
	2	96.7	143	46.3	39,500	0.0012
	3	60.6	114	53.4	38,000	0.0014
	4	100	158	58.0	41,500	0.0014
	5	87.3	113	25.7	29,500	0.0009
Kidney:	No. 1	53.3	53.0	0	12,400	0.0
	2	48.6	60.6	12.0	12,800	0.0009
	3	26.7	40.0	13.3	12,100	0.0012
	4	33.5	33.5	0	12,500	0.0
	5	51.5	80.0	28.5	12,100	0.0024

* Expressed as mμmole thymidine equivalent/1 g wet weight.

** Determined chemically and expressed as mμmole phosphorus/1 g wet weight.

*** See the footnote *** of Table I.

TABLE III

DEOXYNUCLEOSIDIC COMPOUNDS CONTENT OF TUMORS OF THE RAT AND MOUSE

Material	*ASu**	*ASt**	*"Masked" deoxynucleosidic compounds (ASt–ASu)**	*DNA***	*"Masked"§ deoxynucleosidic compounds / DNA*
Rat – Hepatoma 7974					
Solid					
No. 1	156	187	31	6,940	0.0045
2	106	150	44	6,580	0.0077
3	150	164	14	5,830	0.0024
Ascites cells					
No. 1	41	56	15	2,600	0.0058
2	53	62	9	—	—
Ascites fluid**					
No. 1	255	376	121	0	—
2	288	528	240	0	—
Mouse – Sarcoma 180					
No. 1	15	71	52	4,200	0.0124
2	26	71	45	7,570	0.0059
Ehrlich ascites					
cells	6	6	0	2,600	0
fluid**	8	36	24	0	—

* Expressed as mμmole thymidine equivalent/1 g wet weight or ml fluid.
** Determined chemically and expressed as mμmole phosphorus/1 g wet weight.
*** Supernatant fluid obtained by removing the cells by centrifugation.
§ See the footnote *** of Table I.

early embryos during cleavage stage. In this connection, it may be worth noting that during the cleavage stage no great change in the net amount of protein[8] or RNA[9] was found, while DNA shows a remarkable increase.

Data in Table II indicate that the presence of "masked" deoxynucleosidic compounds is not restricted to eggs and embryos but rather widely distributed in adult mammalian tissues including tumors, though their relative amounts compared with simple deoxynucleosidic compounds or DNA in the latter tissues are comparatively small.

Acid-soluble deoxynucleotides of "masked" form were also found in microorganisms and evidence has been presented indicating their active participation in DNA synthesis[11,12,13].

Recently, ROTHERHAM AND SCHNEIDER[15] have reported the presence of "digestible" deoxynucleotides, which are essentially the same as our "masked" deoxynucleosidic compounds, and have come to a conclusion similar to ours about their biological significance.

"Masked" deoxynucleosidic compounds of sea urchin eggs were completely adsorbed on Dowex-1-formate resin from the neutral solution, this indicates that they are acidic and perhaps the derivatives of deoxynucleotides. Further studies on their chemical nature led us to the discovery of a new conjugated derivative of deoxycytidylic acid, deoxycytidine diphosphate choline[14]. Details of these studies and the chemical characteristics of the "masked" deoxynucleosidic compounds will be described in a subsequent paper[16].

ACKNOWLEDGEMENTS

The authors wish to thank Drs. E. OHMURA, M. KAZIWARA, Z. SUZUOKI AND M. KAWASHIMA, and Mr. T. HEMMI for interest and advice. They are also grateful to Professor YAMADA and Professor HAYAISHI for helpful advice on preparing this paper, and to Dr. M. SUGIYAMA for giving facilities for collecting sea urchin eggs and embryos.

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STUDIES ON DEOXYNUCLEOSIDIC COMPOUNDS

II. DEOXYCYTIDINE DIPHOSPHATE CHOLINE IN SEA URCHIN EGGS

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(Received August 18th, 1959)

SUMMARY

1. A new deoxynucleotide derivative was found in sea urchin eggs and was identified as deoxy-CDP-choline.

2. Deoxy-CDP-choline was synthesized chemically.

3. In addition to deoxy-CDP-choline, a large amount of CDP-choline was found in sea urchin eggs.

4. The structural characteristics and biological significance of the "masked" deoxynucleosidic compounds including deoxy-CDP-choline have been discussed.

INTRODUCTION

In the first paper of this series, a modified microbiological assay method for deoxynucleosidic compounds was reported[1,2]. The characteristic feature of the modified method is the digestion of the samples with snake venom enzyme prior to the microbioassay. By applying this method to the acid-soluble extracts of eggs and embryos of the sea urchin and tissues of the rat, a group of deoxynucleosidic compounds was found which would not have been detected if the usual microbiological method had been used. They were tentatively designated as "masked" deoxynucleosidic compounds[1,2] so as to distinguish them from simple deoxynucleosides and deoxynucleotides, which are microbiologically active without any pretreatment[3].

Soon afterwards, one of the "masked" deoxynucleosidic compounds of sea urchin eggs was isolated and identified as deoxy-CDP-choline[4].

This paper is devoted to giving the full details of its isolation, identification, and chemical synthesis. Some discussion of the chemical nature and biological significance of the "masked" deoxynucleosidic compounds is also presented.

METHODS AND MATERIALS

Determination of deoxynucleosidic compounds. Simple and "masked" deoxynucleosidic compounds were assayed microbiologically as previously described[1].

Abbreviations: deoxy-CDP-choline, deoxycytidine diphosphate choline; CDP-choline, cytidine diphosphate choline; CMP, cytidylic acid; deoxy-CMP, deoxycytidylic acid; P-choline, phosphoryl choline; DNA, deoxyribonucleic acid; Dd-CPP, dideoxycytidine pyrophosphate; DCC, dicyclohexyl carbodiimide; AF, ammonium formate; PCA, perchloric acid.

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Determination of choline. Choline was assayed microbiologically using cholineless *Neurospora* mutant[5]. Prior to the microbioassay, samples were digested with snake venom. Conditions for the venom digestion were the same as those used for the assay of "masked" deoxynucleosidic compounds[1].

Ultraviolet-absorption measurement was carried out with a Beckman DU spectrophotometer, using the quartz cell of 1 cm light path.

Phosphorus was determined by the FISKE–SUBBAROW method[6]. Absorption at 660 mμ was read in a Coleman junior spectrophotometer.

Paper chromatography was carried out on Toyo No. 7 or Azumi No. 7 paper using the following solvent systems: (1) 0.02 N acetic acid in 60% ethanol[7]; (2) *n*-propanol–ammonia (d 0.88)–water (7:3:1); (3) saturated $(NH_4)_2SO_4$–water–isopropanol (79:19:2)[8]; (4) ethanol (95%)–ammonium acetate (pH 7.5, 1 M) (7.5:3)[9].

Ultraviolet-absorbing spots were located by Matsuda germicidal lamp (GL-15) fitted with Corning No. 9863 filter. Choline was detected on paper by spraying with Dragendorff reagent[10].

Paper electrophoresis. The following buffers were used: (1) 0.05 M ammonium formate, pH 3.5; (2) 0.05 M sodium phosphate, pH 7.8; (3) 0.05 M sodium borate, pH 9.3.

Ion-exchange chromatography. Dowex-1-formate resins were used. Details of the column operation are described in RESULTS.

Acid-soluble extracts were prepared as described previously[1] from the lyophilized powder of unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*.

The lyophilized egg powder was kindly provided by Dr. R. OKAZAKI of Nagoya University.

Chemicals

Deoxycytidine*, cytidine, deoxy-CMP*, CMP, and choline chloride were commercial preparations. CDP-choline[7] was the gift of Dr. E. P. KENNEDY of University of Chicago. Calcium salt of P-choline was given by Mr. S. IWANAGA of Kyoto University. The calcium salt was converted to the free acid by treatment with Dowex-50-H$^+$. DCC was supplied by Dr. K. OKAWA. of Osaka University.

RESULTS

Ion exchange fractionation of acid soluble deoxynucleosidic compounds of sea urchin eggs

Cold PCA extracts of 800 mg egg powder was neutralized with KOH to pH 8.5. After removal of K-PCA the neutralized extract was passed through a column of Dowex-1-formate. The column was washed with water until the optical density at 260 mμ of the effluent disappeared, and then eluted with various concentrations of AF. The progress of elution was followed by measuring the optical density at 260 mμ (Fig. 1). 14 bulked fractions were obtained by combining tubes as indicated on the abscissa of Fig. 1. Each bulked fraction was concentrated, lyophilized to remove AF, dissolved in a given amount of water, and assayed for the content of deoxynucleosidic compounds. The results are summarized in Table I, which shows that three fractions, Nos. 5, 11 and 13 contained large amounts of "masked" deoxynucleosidic compounds. Of these, Fraction No. 5 aroused interest because its ultraviolet absorption spectra in acid and alkali were very similar to those of CMP. However, in order to decide whether or not the deoxynucleosidic compound contained in Fraction No. 5 is a derivative of deoxy-CMP, further purification using more starting material was needed, since the fraction was undoubtedly contaminated with a large amount of a ribosidic compound(s).

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* Products of California Corporation for Biochemical Research.

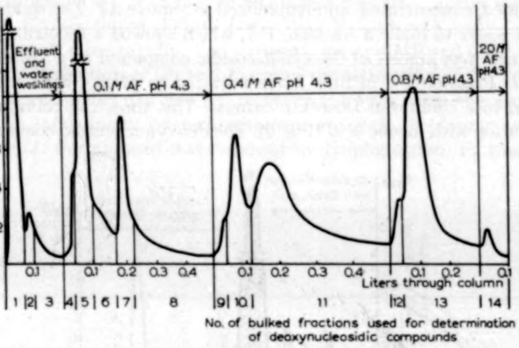

Fig. 1. Ion-exchange fractionation of acid-soluble deoxynucleosidic compounds of sea urchin eggs Sample: neutralized acid-soluble extracts of 800 mg of the egg powder. Exchanger: Dowex-1-formate, X-2, 200–400 mesh, 1 × 24 cm. Elution: as indicated. Amounts of deoxynucleosidic compounds in each bulked fraction are shown in Table I.

TABLE I

AMOUNT OF DEOXYNUCLEOSIDIC COMPOUNDS IN EACH BULKED FRACTION OBTAINED BY ION-EXCHANGE CHROMATOGRAPHY IN STEP I

Fraction No.	*Concn. of AF (M)*	*Deoxynucleosidic compounds*		
		u (before enzyme treatment)*	*t* (after enzyme treatment)*	*t — u* "masked"*
1	0	5	5	0
2		0	5	5
3		0	10	10
4	0.1	0	0	0
5		0	200	200
6		0	28.5	28.5
7		5	21.0	16.0
8		27.5	55.0	27.5
9	0.4	0	25.5	25.5
10		0	10	10
11		22.5	260	237.5
12	0.8	0	0	0
13		0	110	110
14	2.0	0	0	0

* Expressed as mμmole thymidine equivalent.

Purification and isolation of the "masked" deoxynucleosidic compound found in Fraction No. 5

Step I. Acid-soluble extracts of 8.6 g of the lyophilized egg powder, which contained 0.58 μmole and 8.7 μmoles of simple and "masked" deoxynucleosidic compounds respectively, were chromatographed in a similar manner to the above but different in that the column size was enlarged. Tubes corresponding to Fraction No. 5 were combined, concentrated, and lyophilized to remove AF. The residue was dissolved in 50 ml of water to make a fraction, F-I, which showed a spectrum similar to CMP and contained 3.35 μmoles of deoxynucleosidic compound as a "masked" form.

Step II. Fraction F-I of Step I was adjusted to pH 8.5 with aqueous ammonia and applied to a column of Dowex-1-formate. This time, the column was eluted by gradient elution with formic acid (Fig. 2). The deoxynucleosidic compound under consideration was eluted together with the main ultraviolet-absorbing peak F_C (indicated as a shaded area in Fig. 2). F_C showed a CMP-like spectrum and contained 2.49 μmoles of "masked" deoxynucleosidic compound. At this purification stage, however, the amount of the deoxynucleosidic compound estimated microbiologically as deoxycytidine was not more than one-tenth of that calculated from the optical density measurement. This apparent discrepancy indicated that F_C still contained a considerable amount of a ribosidic compound which seemed to be a CMP-derivative. Therefore, to separate effectively the deoxynucleosidic compound from the contaminating ribosidic one, the next purification procedure was designed to utilize a borate system in ion-exchange chromatography, and it was found to be successful.

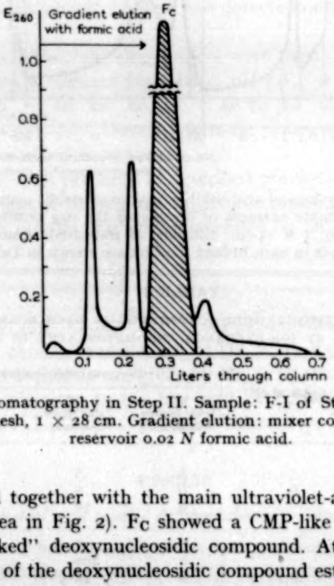

Fig. 2. Ion-exchange chromatography in Step II. Sample: F-I of Step I. Exchanger: Dowex-1-formate, X-2, 200–400 mesh, 1 × 28 cm. Gradient elution: mixer contained 500 ml of water and reservoir 0.02 *N* formic acid.

Step III. Fraction F_C was added to two volumes of water, adjusted to pH 8.5 with aqueous ammonia, and then applied on a column of Dowex-1-formate. All ultraviolet-absorbing materials were adsorbed on the resin. After washing with water, it was eluted by gradient elution with AF, pH 7.8, containing sodium borate, the concentration of the former being gradually increased and that of the latter gradually decreased (Fig. 3). By this procedure, two separate fractions, F_1 and F_2, were obtained, both having spectra similar to CMP. As seen in Fig. 3, microbioassay of each chromatographically separated fraction made it clear that Fraction F_1 was the "masked" deoxynucleosidic compound in question. This result together with the result of spectral measurement suggested that F_1 would be a derivative of deoxy-CMP and F_2 a ribosidic analogue of F_1. It should be mentioned here that, in this ion-exchange chromatography, F_1 was eluted slightly faster than F_2 and both fractions emerged from the column much more rapidly than authentic deoxy-CMP and CMP, indicating the less anionicity of the former (F_1 and F_2) than the latter (Fig. 3).

F_1 and F_2 were further purified by rechromatography with the same borate-containing system, and finally by rechromatography with the formic acid system described in *Step II*. Formic acid was removed by lyophilization. F_1 thus obtained was shown by paper chromatography to be contaminated with a trace amount of F_2, whereas the preparation of F_2 was free of F_1. The yield of the "masked" nucleosidic growth activity in this step was about 80 %.

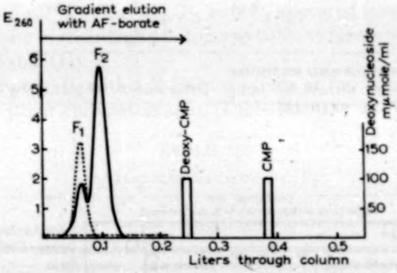

Fig. 3. Ion-exchange chromatography in Step III. Sample: Fc of Step II. Exchanger: Dowex-1-formate, X-8, 200–400 mesh, 1 × 10 cm. Gradient elution: mixer contained 500 ml of 0.0005 *M* sodium borate, pH 7.8 and reservoir 0.5 *M* AF, pH 7.8. ——, chromatogram followed by optical density measurement. - - - - -, chromatogram followed by microbiological measurement of deoxynucleosidic compound after venom digestion. – · – · – · –, the same as above but before venom digestion. The two columns indicate the positions where authentic samples of deoxy-CMP and CMP emerge.

Step IV. The lyophilized preparation of F_1 of Step III was dissolved in a small amount of water, applied on a sheet of Toyo No. 7 paper as a band, and developed with Solvent 1. The main faster band of F_1, located by ultraviolet lamp, was cut out, eluted with water, and lyophilized. This final preparation was completely free of F_2 and migrated as a single spot in paper chromatography and paper electrophoresis carried out under various conditions (see the next section). Its deoxynucleosidic growth activity, determined microbiologically after venom digestion and expressed as mμmoles of deoxynucleoside, showed good agreement with the deoxycytidine content estimated from the optical density measurement (Table III). This means that F_1 is a single compound containing only deoxycytidine as its nucleosidic constituent.

TABLE II

$R_{deoxycytidine}$ VALUES OF F_1, F_2, THEIR DEGRADATION PRODUCTS, AND THE RELATED SUBSTANCES

	Solvent 1		*Solvent 2*	*Solvent 3*	*Solvent 4*
	*u.v.**	*Dragendorff**	*u.v.**	*u.v.**	*u.v.**
Deoxy-CMP	0.50		0.37	1.08	0.04
F_1 after acid hydrolysis	0.49	trailed	—	—	0.03
Deoxycytidine	1.0		1.0	1.0	1.0
F_1 after venom digestion	1.02	1.08	—	—	0.98
F_1	0.35	0.35	0.44	1.12	0.11
Deoxy-CDP-choline	0.34	0.34	0.45	1.13	0.10
CDP-choline	0.25	0.25	0.35	1.22	0.06
F_2	0.25	0.25	0.33	1.21	0.06
F_2 after venom digestion	0.79	1.08	—	—	0.73
Cytidine	0.81		0.78	1.11	0.70
F_2 after acid hydrolysis	0.43	trailed	—	—	0.04
CMP	0.44		0.32	1.19	0.04
Choline		1.07	—	—	—

Solvents: see MATERIALS AND METHODS.

* U.v.: detected under ultraviolet lamp; Dragendorff: detected by spraying Dragendorff reagent (see MATERIALS AND METHODS).

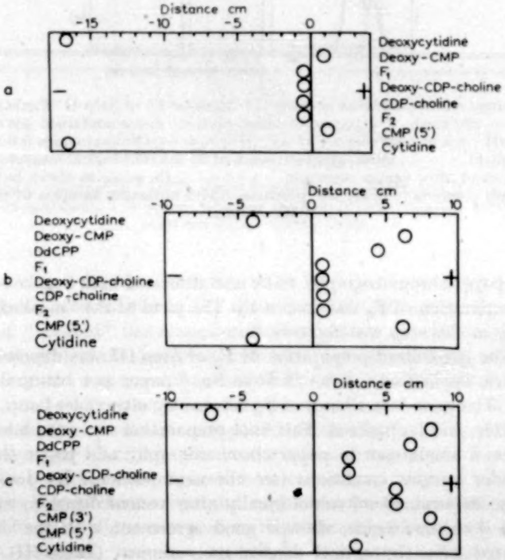

Fig. 4. Paper electrophoresis of F_1, F_2, and the related substances. Conditions: (a) Buffer 1, pH 3.5, 10 V/cm, 7 h. (b) Buffer 2, pH 7.8, 10 V/cm, 3.5 h. (c) Buffer 3, pH 9.3, 10 V/cm, 5.5 h.

Paper chromatography and paper electrophoresis of F_1, F_2, their degradation products, and the related substances

F_1 and F_2 were compared with deoxycytidine, cytidine, CMP, deoxy-CMP, and CDP-choline by paper chromatography and paper electrophoresis. Results are summarized in Table II and Fig. 4. F_2 seemed to be identical with CDP-choline. F_1 appeared to be the deoxyanalogue of F_2, *i.e.* deoxy-CDP-choline, since the positional relationship of F_1 to F_2 (or CDP-choline) was very similar to that of deoxycytidine to cytidine and of deoxy-CMP to CMP.

F_1 as well as F_2 showed a strongly positive spot test on paper with Dragendorff reagent, providing additional evidence for the presence of such a quaternary ammonium as choline in their structure. In fact, the venom digestion of F_1 gave deoxycytidine and choline, and F_2 cytidine and choline, identified by paper chromatography (Table II). The presence of choline residues in F_1 and F_2 fits in well with the fact that, in anion-exchange chromatography at pH 7.8 (Fig. 3) or paper electrophoresis at pH 7.8 (Fig. 4b) and pH 9.3 (Fig. 4c), F_1 and F_2 appeared less anionic than deoxy-CMP and CMP in spite of their high phosphorus content (analytical results are shown in a later section, Table III).

Hydrolysis of F_1 and F_2 with 1 N HCl for 15 min at 100° yielded deoxy-CMP and CMP, identified by paper chromatography (Table II).

TABLE III

ANALYTICAL DATA OF F_1, F_2

Material	*μmole/μmole of deoxycytidine or cytidine**				
	*Deoxyriboside***		*Phosphorus***	*Choline***	
	Before venom treatment	*After venom treatment*		*Before venom treatment*	*After venom treatment*
F_1	0.15	0.93	1.93	0	0.83
F_2	0	0	2.03	0	0.87
Deoxy-CDP-C (synthetic)	0.1	0.98	1.96	—	—

* Estimated from optical density measurement at 260 mμ at pH 2 using $\varepsilon = 6{,}200$.
** See the text.

Microbiological determination of choline

The presence of choline residues in F_1 and F_2 was further confirmed microbiologically using choline-less *Neurospora* as a test organism.

Also in this case, it was noticed that neither F_1 or F_2 supported the growth of the test organism, choline-less *Neurospora*, unless they were hydrolysed with snake venom (Table III). This situation is very similar to that observed in the microbiological determination of deoxynucleoside of F_1, and served as an additional proof that choline is bound to the nucleoside moiety through a linkage sensitive to the venom enzyme.

Analytical data of F_1 and F_2

The absorption spectra of F_1 and F_2, completely coincided with those of deoxy-CMP or CMP. Analytical data of F_1 and F_2 are summarized in Table III. From these data and several lines of evidence already mentioned, it was concluded that F_1 is deoxy-CDP-choline, having a structure shown in Fig. 5, and that F_2 is CDP-choline, which was previously found by Kennedy and Weisss[7,11].

That the structure of this newly found compound, F_1, is that of deoxy-CDP-choline was finally confirmed by its chemical synthesis.

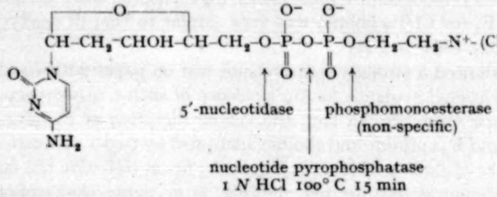
5′-nucleotidase
phosphomonoesterase (non-specific)
nucleotide pyrophosphatase
1 N HCl 100° C 15 min

Fig 5. Structure of F_1, deoxy-CDP-choline. Arrows indicate the positions of cleavage by hydrolysis with the snake venom or acid.

Chemical synthesis of deoxy-CDP-choline

The synthetic method was the same as that described by Kennedy[7] for the synthesis of CDP-choline except that deoxy-CMP was used in place of CMP.

The reaction products obtained from 130 µmoles of deoxy-CMP and 110 µmoles of phosphoryl choline were fractionated by ion-exchange chromatography according to Kennedy. Ultraviolet-absorbing materials were eluted from the column as three peaks, I, II and III, in this order. Each fraction was adsorbed on charcoal, eluted with ammonical ethanol, concentrated under reduced pressure, and lyophilized.

30 µmoles of deoxy-CDP-choline, which gave a strongly positive spot test on paper with Dragendorff reagent, were obtained from peak I. Peak II and III were deoxy-CMP and Dd-CPP, respectively.

Comparison of F_1 with the synthetic deoxy-CDP-choline by paper chromatography (Table II), and paper electrophoresis (Fig. 4), and by chemical and microbiological analyses (Table III) proved that F_1 was deoxy-CDP-choline.

DISCUSSION

Experimental results reported in this paper clearly show that one of the "masked" deoxynucleosidic compounds of sea urchin eggs was deoxy-CDP-choline, which was the first conjugated deoxynucleotide found in nature at the time when the preliminary report appeared[4].

Independently, Potter and Buettner-Janusch discovered deoxy-CDP-ethanolamine in calf-thymus[12]. Soon afterwards, Schneider and Rotherham reported the occurrence of deoxy-CDP-choline and deoxy-CDP-ethanolamine in Novikoff Hepatoma[13]. Okazaki *et al.* found a conjugated thymidylic acid, thymidine diphosphate-X*, in microbial cells[14].

It is of great interest that all of these deoxynucleosidic compounds, which must be digested with snake venom or other enzymes[13] to reveal their full microbiological activity towards *L. acidophilus* R-26, have such a common structural feature that the deoxynucleoside moiety is linked through a pyrophosphate bond to the second component (choline, ethanolamine, etc.). The attachment of pyrophosphate to the deoxynucleoside "masks" the deoxynucleosidic growth activity, and such enzymes as cleave the pyrophosphate bond to give a deoxynucleoside or deoxynucleoside monophosphate can be used to activate the "masked" deoxynucleotides. Thus, the action of snake venom, which liberated deoxycytidine and choline from deoxy-CDP-choline, can be explained by its nucleoside pyrophosphatase[15–18] and phosphomonoesterase[19–22] (including 5′-nucleotidase) activities.

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* Recently X has been identified as rhamnose[26].

This view coincides well with the fact that di- or tri-phosphates of pyrimidine deoxynucleosides do not support the growth of *L. acidophilus* R-26 unless they are dephosphorylated to monophosphates[23], and that chemically synthesized Dd-CPP, which was microbiologically inactive, could support the growth of *L. acidophilus* when it was hydrolysed with snake venom[24]. Similar results were reported by Rotherham and Schneider[25], who used intestinal phosphatase instead of the snake venom[*].

The biochemical function(s) of deoxy-CDP-choline and other "masked" deoxynucleotides is as yet unknown. However, their coenzyme-like structure and distribution strongly suggest that they must actively participate in some biosynthetic reactions of rapidly dividing cells or cell nuclei, where rapid DNA synthesis proceeds.

Further investivation is needed in order to decide what specific functions the conjugated deoxynucleotides, including deoxy-CDP-choline, may carry out.

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Drs. S. Kuwada, E. Ohmura, Z. Suzuoki and K. Kawashima, Mr. T. Henmi, and Professor Hayaishi for their interest and valuable advice. He is also grateful to Dr. R. Okazaki for collecting sea urchin eggs, to Dr. E. P. Kennedy for a sample of CDP-choline, to Dr. K. Okawa for a sample of DCC, to Mr. S. Iwanaga for a sample of P-choline and to Mrs. Sugino for her able technical assistance.

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[*] Our venom preparations, vacuum-dried powder of venoms of *Trimeresurus flaroviridus* and *Aghistrodon blomhoffi*, did not contain appreciable nucleosidase activity, while cobra venom was found to contain the nucleosidase activity[25].

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INFLUENCE DE DIVERS AGENTS CHIMIQUES SUR LA CAROTÉNOGÉNÈSE DE *RHODOTORULA MUCILAGINOSA*

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(Reçu le 2 septembre, 1959)

SUMMARY

***The influence of various chemical agents on the carotenogenesis of* Rhodotorula mucilaginosa**

The action of phenol, phenylethylacetamide, acenaphthene, naphthalene, phenantrene, streptomycin, and diphenylamine on the carotenogenesis of *Rh. mucilaginosa* was studied; where the action was inhibitory, it was accompanied by a decline in the growth of the cultures: a fact that indicates the complex nature of the phenomenon.

Biphenyl incorporated in the culture medium at a concentration of 50 μg/ml has practically no effect on the growth of *Rh. mucilaginosa*, and permits the obtention of a white yeast. Among the diphenyl derivatives studied, only the asymmetrically substituted ones inhibited the carotenogenesis.

INTRODUCTION

Une des méthodes permettant d'aborder le mécanisme de la biosynthèse des caroténoïdes, réside dans l'utilisation d'inhibiteurs spécifiques. Ce procédé largement employé par de nombreux auteurs et chez divers microorganismes[1] ne semble pas,

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* Directeur: Professeur F. KAYSER.

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en ce qui concerne les levures rouges, avoir connu une pareille extension. Nous rapportons ici les résultats que nous avons obtenus dans l'étude de l'inhibition de la caroténogénèse de *Rh. mucilaginosa*.

Dans nos expériences nous nous sommes adressé d'une part à des agents dont l'action inhibitrice sur la biosynthèse des caroténoïdes était reconnue (phénol, diphénylamine, etc....) d'autre part, à des corps pour lesquels nous avons mis en évidence une action anticaroténogène.

PARTIE EXPERIMENTALE

Matériel et techniques

Microorganisme: La levure utilisée au cours de ce travail a été isolée accidentellement à partir d'une levure de brasserie récoltée dans des filtres-presses.

Il s'agit d'une levure appartenant à l'espèce *Rhodotorula mucilaginosa**.

Techniques de culture: Une fiole d'Erlenmeyer de 100 ml contenant 20 ml de milieu est ensemencée à partir d'une culture sur gélose inclinée. La fiole est portée dans un thermostat à 30°, l'aération est réalisée par agitation magnétique.

Le milieu utilisé est entièrement synthétique, en voici la composition:

Thiamine (chlorhydrate) 0.4 mg; riboflavine 0.2 mg, acide nicotinique 5 mg, acide *p*-aminobenzoïque 0.3 mg, pyridoxal 1 mg, pantothénate de calcium 0.5 mg, méso-inositol 50 mg, biotine 0.002 mg, L-histidine (monochlorhydrate) 10 mg, L-tryptophane 20 mg, DL-méthionine 20 mg, sulfate d'ammonium 3 g, phosphate monopotassique 2 g, sulfate de magnésium 7 H_2O 0.25 g, chlorure de calcium 6 H_2O 0.25 g, chlorure de manganèse 4 H_2O 1 mg, sulfate zinc 7 H_2O 1 mg, acide borique 1 mg, chlorure ferrique 0.5 mg, sulfate de cuivre 5 H_2O 1 mg, iodure de potassium 1 mg, saccharose 20 g, eau distillée Q.S.p 1000 ml.

Stérilisation 20 min à l'autoclave à 120°. pH 5.2.

Incorporation des inhibiteurs au milieu de culture: Pour les corps insolubles dans l'eau, nous avons utilisé comme "solubilisant" le Tween 80 dont quelques essais préliminaires nous ont montré la parfaite inocuité sur la croissance et la pigmentation de *Rh. mucilaginosa* dans nos conditions expérimentales.

Nous avons pu ainsi obtenir des liquides contenant jusqu'à 2 mg/ml d'hydrocarbure qui, dans la plupart des cas restaient limpides.

Estimation de la croissance: Elle a été effectuée à l'aide d'un spectrophotomètre Coleman junior, par la mesure de la densité optique de la suspension à 610 mμ.

RÉSULTATS

Phénol

TURIAN[2] a mis en évidence son action puissante sur la caroténogénèse de *Mycobacterium phlei;* il ne semble pas toutefois qu'il s'agisse là d'une propriété générale puisque le phénol est sans action, par exemple, sur *Phycomyces*[3]. De plus la souche de *Mycobacterium* utilisée par TURIAN paraît avoir présenté une sensibilité particulière[4].

Nous avons essayé ce corps sur *Rh. mucilaginosa*, les résultats ont été difficiles à interpréter car il ne nous a pas été possible de dissocier l'action sur la croissance de celle sur la caroténogénèse.

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* Le Centraal Bureau voor Schimmelcultures-Yeast Division à Delft a confirmé l'identité de cette souche avec *Rhodotorula mucilaginosa* (Jörgensen) Harrison.

Phényléthylacétamide

Ce composé commercialisé sous le nom d'hypostérol est utilisé en thérapeutique comme hypocholestérolémiant. Malgré de nombreux travaux, le mode d'action de cette amide reste hypothétique, il semble cependant que ce soit la synthèse de l'acétyl CoA ou de l'acétoacétyl CoA qui soit inhibée[5]. Nous avons pensé que l'hypostérol pourrait être un inhibiteur de la caroténogénèse si, comme cela a été avancé, l'acétate en était bien le précurseur lointain.

En fait pour des concentrations inférieures à 1 mg/ml la croissance et la caroténogénèse ne sont pratiquement pas perturbées. Pour des concentrations de 1 à 2 mg/ml l'inhibition de la caroténogénèse va de pair avec celle de la croissance.

La levure est à la fois moins abondante et moins colorée rendant donc difficile l'analyse du phénomène.

Hydrocarbures à noyaux condensés

Ark[6] étudiant l'action du naphtalène, de l'acénaphtène et de leurs dérivés sur la chromogénèse de *Corynebacterium michiganense* et de *Xanthomonas juglandis*, semble avoir obtenu dans quelques cas de véritables mutations tandis que dans les autres cas, il s'agit vraisemblablement d'une simple inhibition de la chromogénèse.

En utilisant ces observations comme idée directrice, nous avons pensé tester l'action de divers hydrocarbures sur la caroténogénèse: naphtalène, acénaphtène, phénanthrène. L'action sur la croissance a été relativement faible comme l'indique le Tableau I.

TABLEAU I

ACTION SUR LA CROISSANCE DE *Rh. mucilaginosa* D'HYDROCARBURES À NOYAUX CONDENSÉS

Inhibiteur 200 μg/ml	*Densité optique × 100, mesurée au bout de*						
	12 h	*14 h*	*16 h*	*18 h*	*20 h*	*24 h*	*38 h*
Témoin	50	64	67	83	88	96	105
Acénaphtène	32	41	54	64	75	89	105
Naphtalène	38	49	63	73	83	93	103
Phénanthrène	45	57	70	79	86	95	101

L'acénaphtène non seulement n'a manifesté aucune action inhibitrice mais a toujours conduit, après plusieurs essais à une levure plus colorée que le témoin même pour des concentrations atteignant $4 \cdot 10^{-3}$.

Le naphtalène retarde simplement l'apparition de la pigmentation qui ne commence à être visible qu'à partir de la 16ème heure lorsque la densité optique de la culture atteint déjà 63 alors que la coloration du témoin est déjà parfaitement perceptible pour une densité optique de 30 environ. En fin de croissance, la levure ayant poussé en présence de naphtalène présente une teinte et une intensité de coloration comparables à la culture témoin.

Le phénanthrène, comme le naphtalène, retarde l'apparition de la pigmentation mais la levure présente en fin de croissance une coloration rose orangé que nous n'avions jamais obtenue jusqu'alors.

Ces résultats bien que présentant un certain intérêt, puisque deux des trois corps essayés ont certainement modifié la caroténogénèse, ne nous ont pas paru assez nets pour justifier, pour l'instant, une étude plus approfondie car nous avons trouvé dans la série du biphényle des inhibiteurs beaucoup plus actifs.

Streptomycine

Chez *Phycomyces blakesleeanus*, la streptomycine agit sélectivement sur la caroténogénèse mais uniquement si la source azotée est l'asparagine. En effet, si on remplace l'asparagine partiellement ou totalement par un sel d'ammonium la streptomycine n'agit plus. De même dans un milieu contenant de l'acétate sans glucose, la streptomycine inhibe d'une façon identique la croissance et la caroténogénèse de *Phycomyces blakesleeanus*, si bien que la concentration en pigment reste constante[7].

Chez *Rhodospirillum rubrum* bien qu'inhibant la croissance, cet antibiotique n'a pratiquement pas d'action sur la pigmentation[8].

Par contre, chez une Euglène sp., la destruction des chloroplastes par la streptomycine est accompagnée d'une inhibition marquée mais non totale de la synthèse des caroténoïdes[9].

L'utilisation de la streptomycine comme inhibiteur de la pigmentation des levures rouges a fait l'objet d'une étude de BERTOSSI ET CIFERRI chez *Rh. rubra*[10]. Ces auteurs trouvent que la streptomycine, à des concentrations allant de 10^{-5} à 10^{-3}, est sans action sur la croissance comme sur la teneur en torularhodine, torulène, β-carotène, et γ-carotène.

Les expériences que nous avons réalisées avec *Rh. mucilaginosa* montrent que cette levure est très peu sensible à la streptomycine ce qui ne nous a pas surpris, les levures étant très résistantes aux antibiotiques. Que la source azotée soit sous forme d'asparagine ou de sulfate d'ammonium, ni la croissance, ni la caroténogénèse n'ont été modifiées pour des concentrations en antibiotique allant de $2 \cdot 10^{-5}$ à $2 \cdot 10^{-2}$.

Diphénylamine (*DPA*)

KARASCH, CONWAY ET BLOOM[11] avaient montré que la chromogénèse est inhibée chez plusieurs bactéries et champignons par DPA. TURIAN[12] reprenant ces observations montre que cette substance, à la concentration de 1/20,000 à 1/35,000 inhibe la caroténogénèse de *Mycobacterium phlei*. GOODWIN[13] a étudié l'action de cette amine sur *Phycomyces blakesleeanus* et a pu montrer que la production des α-, β- et γ-carotènes et celle du lycopène est pratiquement abolie tandis que la teneur en phytoène, phytofluène, ζ-carotène et neurosporène est nettement augmentée. Bien que de nombreux chercheurs aient utilisé DPA pour inhiber la caroténogénèse, le mode d'action de ce composé est resté inconnu; d'ailleurs, le métabolisme des caroténoïdes n'est problablement pas seul à être perturbé car, dans bien des cas, la croissance a été fortement diminuée sinon totalement empêchée. GOODWIN[14] signale par exemple l'inhibition totale de la croissance de *Chlorella vulgaris* par DPA à 1/280,000.

A notre connaissance, peu de travaux signalent l'utilisation de DPA dans l'étude de la caroténogénèse chez les levures appartenant au genre *Rhodotorula*.

SLECHTA *et al.*[15] ont montré que dans le cas de *Rhodotorula gracilis*, DPA à la concentration de $4.5 \cdot 10^{-5}$ inhibe totalement la synthèse des pigments.

Pour *Rhodotorula rubra*, H. WHITTMAN[16] trouve également une inhibition de la caroténogénèse par DPA sans que la croissance soit influencée pour une concentration de 1 % en inhibiteur (!). La levure dans ce cas présente des cellules beaucoup plus grandes que les témoins.

. Nous avons de notre côté, testé la sensibilité de *Rhodotorula rubra** à DPA, la croissance est très fortement diminuée pour des concentrations de 4 à $5 \cdot 10^{-5}$.

Sur notre souche de *Rh. mucilaginosa*, DPA exerce une puissante action anticaroténogène, dès la concentration de 10^{-5}; l'inhibition ne semble totale que pour des concentrations de 4 à $5 \cdot 10^{-5}$.

Le Tableau II montre que l'inhibition de la caroténogénèse est accompagnée d'une inhibition de la croissance ce qui prouve que DPA agit sur plusieurs mécanismes.

Plusieurs auteurs ont obtenu, dans des conditions différentes, la levée de l'inhibition produite par DPA.

Les cultures de *Phycomyces blakesleeanus* en présence de DPA voient leur taux de β-carotène redevenir sensiblement normal si on ajoute au milieu, de la riboflavine ou de l'acide adénylique de muscle; l'acide adénylique de la levure est sans action[3].

TABLEAU II

ACTION DE DPA SUR LA CROISSANCE DE *Rhodotorula mucilaginosa*

Concentration en DPA en µg/ml	*Densité optique × 100, mesurée au bout de*									
	15 h	*17 h*	*19 h*	*21 h*	*24 h*	*40 h*	*43 h*	*62 h*	*65 h*	*72 h*
0	66	77	85	95	103	120				
10	48	62	73	83	93	114				
20	21	33	48	63	79	110				
50	0	0	0	1	4	37	43	59	56	60

Dans le cas de *Rhodospirillum rubrum* par contre, la riboflavine et l'adenosine-5-phosphate sont incapables de ramener la caroténogénèse. Cependant l'action de DPA n'est pas irréversible car si après élimination de cet inhibiteur par des lavages, on place la masse bactérienne dans un tampon phosphate, une pigmentation voisine de la normale peut être obtenue aussi bien en anaérobiose en présence de lumière qu'en aérobiose dans l'obscurité[17, 18].

Rhodotorula gracilis représente un cas bien différent[15]: si après croissance en présence de DPA, cette levure est lavée puis placée dans un milieu ne contenant ni DPA, ni source azotée, la caroténogénèse reste inhibée alors que la levure normale donne bien dans les mêmes conditions une nette synthèse de caroténoïdes. Si au milieu de culture précédent, on rajoute une source azotée et laisse la levure proliférer, cette dernière recouvre peu à peu sa capacité de synthétiser les pigments; *Rhodotorula rubra* semble se comporter d'une façon semblable[16].

Nous avons également tenté pour *Rhodotorula mucilaginosa* de lever l'inhibition de la caroténogénèse provoquée par DPA.

Une culture ayant poussé sur 20 µg/ml de DPA est lavée 4 fois avec du phosphate monopotassique $M/15$ puis remise en suspension dans ce tampon. Après 20 h d'aération intense la culture reste blanche; dans ces conditions, la synthèse endogène des pigments n'est donc pas réalisable. L'apport d'une source exogène de carbone sous forme de saccharose ou de glucose n'a pas donné plus de résultat.

Il a fallu pour obtenir la levée d'inhibition permettre à la levure de se reproduire par repiquage sur son milieu habituel et même dans ces conditions la caroténogénèse reste très faible jusqu'au 5ème repiquage environ. Ce fait laisse penser que DPA reste très fortement fixée sur les cellules et la synthèse des pigments n'a lieu qu'au fur et à mesure de la dilution de l'inhibiteur par suite de la multiplication cellulaire. De sorte que nous n'assistons pas à une levée d'inhibition mais à une dilution de l'inhibiteur jusqu'à atteindre la teneur où il devient inactif.

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* Souche CBS No. 109 fournie par le Centraal Bureau voor Schimmelcultures à Delft.

Biphényle

L'action de ce composé sur la caroténogénèse de ***Rhodotorula mucilaginosa*** est intense car pour des concentrations de l'ordre de 20 µg/ml de milieu, on obtient déjà une levure à peine colorée* dont la croissance est cependant identique à celle du témoin.

Pour une concentration de 50 µg/ml la levure obtenue est aussi blanche que celle résultant de l'action de DPA et ce, pour des concentrations de même ordre de ces 2 agents. Le biphényle, dans nos conditions expérimentales, présente de plus l'avantage qu'à concentration égale, la croissance est inchangée, en tous cas beaucoup moins affectée que dans le cas de l'emploi de la diphénylamine (voir Tableaux II et III).

TABLEAU III

ACTION DU BIPHÉNYLE SUR LA CROISSANCE DE *Rhodotorula mucilaginosa*

Concentration du biphényle en µg/ml	*Densité optique × 100, mesurée au bout de*								
	11 h	*13 h*	*14 h*	*15 h*	*16 h*	*18 h*	*20 h*	*22 h*	*24 h*
0	22	30	36	42	50	68	81	90	103
50	13	21	27	35	41	57	72	84	92
100	13	21	26	36	41	59	74	84	93
140	11	18	23	31	36	53	67	78	86
200	11	18	22	30	37	52	67	78	86

Derivés du biphényle

Devant ce résultat encourageant, il nous a paru intéressant de voir si l'activité du biphényle pouvait être encore exaltée par l'introduction de substituants dans la molécule.

Les hydrocarbures s'oxydant, dans les milieux biologiques, généralement en phénols nous pouvions penser que le biphényle est transformé également en dérivés phénoliques par la levure. En ajoutant ces derniers au milieu de culture, nous devions constater une action beaucoup plus marquée que celle de l'hydrocarbure.

D'un autre côté, bien que le mécanisme d'action de DPA ne soit pas connu il est probable que la fonction amine n'est pas étrangère au phénomène, aussi avons nous pensé que les aminobiphényles pouvaient présenter une action supérieure à celle de l'hydrocarbure lui-même.

Nous avons soumis à l'expérimentation les composés suivants: 2-hydroxybiphényle, 2,2'-dihydroxybiphényle, 4-hydroxybiphényle, acide diphénique, 4-aminobiphényle, 4,4′-diaminobiphényle et enfin 4,4′-diamino-3,3′-diméthylbiphényle (tolidine).

---

* A l'encontre des observations de ARK[6] et malgré de très nombreux isolements et contrôles microscopiques, nous n'avons jamais constaté de modification de notre souche, pouvant laisser supposer que les hydrocarbures ou dérivés employés dans nos expériences, avaient provoqué des mutations.

Nous avons suivi l'action de ces corps en fonction de la concentration sur la caroténogénèse et sur la croissance de la levure.

Nos résultats sont résumés dans le Tableau IV. La pigmentation est exprimée par +++ pour une levure possédant une intensité de pigmentation identique à celle de la souche mère, cultivée dans les mêmes conditions, mais en l'absence d'inhibiteur.

TABLEAU IV

ACTION SUR LA CROISSANCE ET LA CAROTÉNOGÉNÈSE DE *Rhodotorula mucilaginosa*, DES DÉRIVÉS DU BIPHÉNYLE

Inhibiteur	*Concentration en µg/ml*	*Pigmentation*	*Densité optique au bout de 24 h*
2-hydroxybiphényle	5	+	105
	10	0	100
	20	0	89
	50	0	67
2,2′-dihydroxybiphényle	20	+++	95
	50	+++	80
	100	++	70
4-hydroxybiphényle	10	0	95
	20	0	62
	50	0	8
	100	0	6
Acide diphénique	50	+++	100
	100	+++	100
	200	+++	100
	400	+++	100
4-aminobiphényle	20	+	100
	50	0	85
	100	0	42
4,4′-diaminobiphényle	100	+++	125
	200	+++	125
Tolidine	20	+++	115
	50	+++	115
	100	+++	110
	200	+++	120
	300	+++	120
	500	+++	125
Témoin	—	+++	110

Il en ressort clairement que certains dérivés du biphényle possèdent un pouvoir inhibiteur élevé de la caroténogénèse dans nos conditions expérimentales. Ce sont: le 2-hydroxybiphényle, le 4-hydroxybiphényle, le 4-aminobiphényle.

D'autres dérivés sont totalement dépourvus d'action sur la pigmentation et certains d'entre eux semblent favoriser très légèrement la croissance de la levure.

Ce sont: le 2,2'-dihydroxybiphényle, l'acide diphénique, le 4,4'-diaminobiphényle, la tolidine.

D'après ces résultats, il est possible de voir que parmi les composés du biphényle essayés, tous les dérivés possédant une molécule symétrique sont dépourvus d'action sur les deux phénomènes: croissance et caroténogénèse alors que tous les dérivés dissymétriques possèdent une puissante action anticaroténogène. Il s'agit peut-être là d'une coïncidence qu'il est prématuré de généraliser.

Nous avons tenté de lever l'action inhibitrice des dérivés du biphényle en mettant à profit l'observation de WEST[19] que, chez le rat, la toxicité du biphényle est diminuée par addition de méthionine au régime. Bien que cette action soit indirecte, nous avons testé la méthionine et la cystéine à des concentrations allant de 40 à 200 μg/ml.

De tous nos essais, seule l'action de 4-aminobiphényle est abolie par l'addition de cystéine mais non de méthionine au milieu de culture.

RÉSUMÉ

L'action du phénol, de la phényléthylacétamide, de l'acénaphtène, du naphtalène, du phénanthrène, de la streptomycine, de la diphénylamine, sur la caroténogénèse de *Rh. mucilaginosa* a été étudiée: lorsqu'une action inhibitrice s'exerce, elle s'accompagne d'une diminution de la croissance des cultures ce qui indique la complexité du phénomène.

Le biphényle, incorporé dans le milieu de culture à la concentration de 50 μg/ml, n'agit pratiquement pas sur la croissance de *Rh. mucilaginosa* et permet d'obtenir une levure blanche. Parmi les dérivés du biphényle étudiés, seuls ont présenté une action inhibitrice de la caroténogénèse, ceux qui sont substitués de façon dissymétrique.

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LES CAROTÉNOÏDES DE *RHODOTORULA MUCILAGINOSA* ÉTUDE DE LEUR BIOSYNTHÈSE A L'AIDE DE L'ANALYSE DE MUTANTS ET DE L'EMPLOI D'UN INHIBITEUR DE LA CAROTÉNOGÉNÈSE

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(Reçu le 2 Septembre, 1959)

SUMMARY

The carotenoids of Rhodotorula mucilaginosa. *A study of their biosynthesis by means of mutant analysis and by use of an inhibitor of carotenogenesis*

1. The strain of *Rhodotorula mucilaginosa* studied contains the following carotenoids, in μg/g of dry substance: torularhodine 98, torulene 16, γ-carotene 1.5, β-carotene 5.5. It contains neither phytoene nor phytofluene.

2. By repeated u.v. irradiations stable strains were isolated, the composition of which differs greatly both in quality and quantity from that of the mother strain: the proportion of some carotenoids is lower or even zero, but it can also be higher, and sometimes carotenoids that have not been found in the mother strain appear.

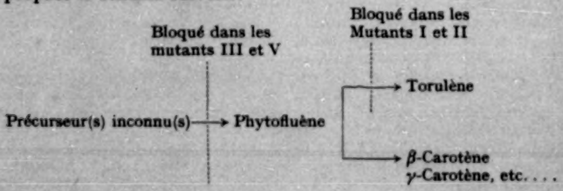
3. Grown in the presence of 2-hydroxybiphenyl, *Rh. mucilaginosa* becomes white, but it still contains small quantities of polyenes. The inhibitor can be removed by washing and an endogenous synthesis of pigments can be revealed.

4. The results obtained do not seem consistent with the idea that the different polyenes are mutually related.

INTRODUCTION

Si de nombreux auteurs ont étudié la nature des caroténoïdes des levures du genre *Rhodotorula*, peu d'entre eux ont tenté d'utiliser ces microorganismes pour aborder le mécanisme de la biosynthèse de ces pigments.

Bonner *et al.*[1] après avoir obtenu des mutants de *Rh. rubra* par irradiation ultraviolette ont proposé le schéma suivant:



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NAKAYAMA *et al.*[2] ont montré que le torulène et la torularhodine se synthétisent probablement aux dépens du β-carotène chez *Rh. glutinis.*

Dans le présent travail qui porte sur *Rh. mucilaginosa* nous étudions d'une part les modifications de la caroténogénèse provoquées par des mutations dues à des irradiations u.v. et d'autre part celles apportées par l'emploi d'un inhibiteur. L'analyse des résultats obtenus nous permet de proposer un schéma de la biosynthèse des caroténoïdes chez *Rh. mucilaginosa.*

MATÉRIEL ET MÉTHODES

Microorganisme

Levure appartenant à l'espèce *Rhodotorula mucilaginosa* (Jörgensen) Harrison et identifiée par Delft.

Milieu de culture: Il s'agit d'un milieu semi-synthétique où l'apport de facteurs de croissance se fait sous forme d'un "jus de levure" obtenu selon CONWAY ET BREEN[3]: 25 g de levure de boulangerie sont dispersés dans 250 ml d'eau distillée, le tout est porté à ébullition que l'on maintient pendant 1 h, puis centrifugé; le surnageant constitue le "jus de levure".

La formule du milieu est la suivante: sulfate d'ammonium 3 g, phosphate monopotassique 2 g, sulfate de magnésium 7 H_2O 0.25 g, chlorure de calcium 6 H_2O 0.25 g, chlorure de manganèse 4 H_2O 0.001 g, sulfate de zinc 7 H_2O 0.001 g, acide borique 0.001 g, chlorure ferrique 0.0005 g, sulfate de cuivre 5 H_2O 0.001 g, iodure de potassium 0.001 g, saccharose 20 g, jus de levure 250 ml, eau distillée Q.S.p 1,000 ml. Stérilisation 20 min à l'autoclave à 120°. Le pH est alors 5.2–5.4.

Mode de culture

Pratiquement toutes nos cultures ont été réalisées en boîte de Roux sur milieu semi-synthétique additionné de 20 g de gélose/l. Après ensemencement, les boîtes sont laissées à température ambiante pendant 4 jours sans prendre de précautions spéciales contre la lumière.

Irradiation ultraviolette

A l'irradiation des cellules en suspension dans un liquide agité, nous avons préféré la méthode suivante plus facile à mettre en oeuvre:

La levure est mise en suspension dans un peu d'eau distillée stérile puis diluée de façon à obtenir environ 1,000 cellules par ml. Un volume connu de cette dilution est étalé à la surface d'un milieu nutritif coulé en boîte de Pétri.

Le générateur d'ultraviolet que nous utilisons est du type Germicide, il se présente sous la forme d'un tube de 110 mm de longueur et de 5 mm de diamètre extérieur (tube 123–100, Anciens Etablissements Gallois et Cie, Paris), fonctionnant sous haute tension et basse pression. Le spectre d'émission de ce tube est surtout représenté par la raie 2537 Å. Le générateur est mis en fonctionnement 1 h avant le début de chaque irradiation de façon à obtenir une émission stable et d'autre part à assurer une stérilisation suffisante de l'espace où se feront les manipulations.

Nous portons la boîte de Pétri à environ 10 cm de la lampe et enlevons le couvercle qui est laissé dans le champ de la lampe pendant la durée de l'irradiation.

Nous replaçons ensuite le couvercle sur la boîte qui est portée à l'étuve à 30°

pendant 48 h. Une boîte de Pétri préparée dans les mêmes conditions, mais non irradiée, sert de témoin. Sauf indication contraire, le temps d'irradiation, déterminé par des essais préliminaires, a été de 4 min.

Extraction des caroténoïdes

Après lavage à l'eau distillée et centrifugation, la levure est traitée par de l'acétone à froid qui extrait, dans le plupart des cas, la presque totalité des caroténoïdes. Lorsque l'action de ce solvant est épuisée, nous achevons l'extraction par le mélange acétone–méthanol (3 : 1) qui laisse une levure blanche.

Cependant certaines cultures fournissent des levures dont les caroténoïdes ne peuvent pratiquement pas être extraits par le procédé ci-dessus. Une observation fortuite nous a montré que si la masse humide est agitée avec du benzène pur (qui dans ces conditions n'extrait pas de pigment), le traitement ultérieur par le mélange acétone–méthanol, après élimination du benzène, permet l'extraction totale des caroténoïdes. Ce dernier procédé a cependant l'inconvénient de donner des émulsions au moment des lavages.

Quel que soit le procédé d'extraction utilisé, les solvants sont réunis, additionnés d'éther de pétrole dans lequel on fait passer le pigment à l'aide d'une solution aqueuse à 20 % de chlorure de sodium. L'éther de pétrole, éventuellement le mélange éther de pétrole–benzène, est lavé à l'eau puis séché sur sulfate de sodium anhydre. On distille ensuite à sec en atmosphère d'azote à l'aide d'une pompe à vide à injection d'air.

Le résidu est repris par de l'éther de pétrole et la solution est placée au frigidaire pendant une nuit ce qui précipite la majeure partie des stérols qui sont éliminés par centrifugation à 0° (la filtration a dû être proscrite car une partie du pigment reste fixée sur le filtre, même si celui-ci est en verre fritté).

Chromatographie et dosage des caroténoïdes

Après élimination des stérols, la solution éthéropétrolique du pigment brut est versée avec précaution sur une colonne d'alumine Merck désactivée selon GOODWIN[4]. Pour certaines fractions, une deuxième chromatographie sur magnésie a été nécessaire. Tous les solvants ont été soigneusement redistillés et l'absence de fluorescence contrôlée dans l'u.v.

TABLEAU I

DONNÉES SPECTROGRAPHIQUES POUR LE DOSAGE DES CAROTÉNOÏDES DE *Rh. mucilaginosa*

Caroténoïde	*Position des maxima d'absorption dans l'éther de pétrole en mμ*			$E^{1\%}_{1\,cm}$ *pour le maximum principal*	*Référence*
Phytoène	275	285	296	850	5
Phytofluène	332	348	367	1,540	6
β-Carotène	424	450	477	2,590	4
ζ-Carotène	378	399	424	2,500	7
γ-Carotène	435	460	491	2,760	8
Neurosporène	415	439	469	2,990	9
Lycopène	446	474	506	3,460	12
Torulène	458	484	518	2,680	10
Spirilloxanthine	466	494	528	2,360	13
Torularhodine	474	500	533	2,580	11

Pour le dosage des caroténoïdes, nous avons utilisé les coefficients d'extinction suivants: (voir Tableau I) définis par la relation

$$E_{1\,\text{cm}}^{1\,\%} = \frac{D \times 10}{C}$$

où $D =$ densité optique

$C =$ concentration en μg/ml.

Toutes les mesures ont été faites dans l'éther de pétrole à l'aide d'un spectrophotomètre Beckman modèle D.U.

RÉSULTATS

Obtention de mutants sous l'action de l'irradiation u.v.

A partir d'une de nos cultures conservée sur milieu semi-synthétique, nous avons constitué une souche pure de *Rh. mucilaginosa* par isolement d'une seule cellule au micro-manipulateur. Cette souche mère sera désignée dans la suite du travail par RMP.

En soumettant cette dernière à l'action de l'u.v. dans les conditions exposées ci-dessus, nous avons obtenu, avec un taux de mortalité de 98%, des colonies rose pâle ne présentant entre elles aucune différence macroscopique. Une de ces colonies est prélevée, une fraction en est repiquée sur milieu solide et constitue la souche RIN, l'autre fraction sert à préparer les dilutions qui subiront une nouvelle irradiation.

Celle-ci permet d'obtenir au bout de 48 h une colonie jaune (RJ1), une colonie orange (RO1) et une dizaine de colonies blanches dont une seule est conservée. A partir de ces différentes colonies, des souches pures sont obtenues en isolant une cellule au micromanipulateur. Il faut noter que, dans cette expérience, le taux de mortalité n'a été que de 80% au lieu de 98% dans l'irradiation précédente.

A ce stade de nos recherches, nous avons pensé que l'atténuation de la pigmentation des levures sous l'influence de l'irradiation était en quelque sorte un phénomène progressif, des cellules étaient devenues blanches parce que le mécanisme de leur caroténogénèse avait subi une atteinte plus profonde que les cellules jaunes, celles-ci ayant été plus sérieusement atteintes que les cellules orange.

Pour tenter de le démontrer, nous avons réirradié la souche RJ1 dans les mêmes conditions, avec la pensée que nous obtiendrions des colonies blanches; cependant sur 3,000 cellules ensemencées, nous n'avons décelé, après une nouvelle exposition aux u.v. identique aux précédentes, aucune colonie blanche. Nous avons prélevé une colonie au hasard qui, après culture, nous a donné la souche RJ2. Cette dernière a été, à son tour, soumise aux u.v. sans modification notable de la pigmentation et une colonie a été cultivée, elle a donné la souche RJ3.

L'irradiation de cette nouvelle souche RJ3 aboutit à l'obtention d'une seule colonie jaune pâle (RJP) au milieu de colonies apparemment inchangées. Ce résultat nous confirmait dans la pensée que l'irradiation aboutit à une décoloration progressive mais si une nouvelle exposition à l'u.v. de RJP nous a permis d'observer une colonie jaune très pâle (RJPX) et une cinquantaine de colonies blanches, dont l'une constituera la souche M1, la présence d'une colonie orange (OR1) venait infirmer notre hypothèse: l'irradiation pouvait aussi parfois augmenter la pigmentation aussi bien que la diminuer.

Ce résultat nous a incité à poursuivre les irradiations afin d'essayer d'obtenir une souche dont la pigmentation serait aussi voisine que possible de celle de la souche mère.

OR1 a donc été soumis à l'irradiation dans les mêmes conditions que les souches précédentes et, après deux tentatives infructueuses, nous avons obtenu: deux colonies dont l'une, rose orangé, constituera la souche RJPY et l'autre, orangé foncé, la souche RJPZ.

Toutes ces souches, dont nous donnons ci-dessous le schéma de filiation sont stables depuis leur isolement qui date, en moyenne, de quatre ans.

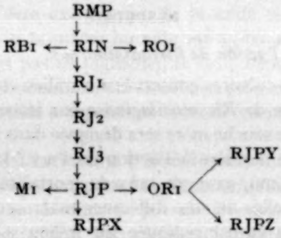

Schéma résumant la filiation des différentes souches venant d'être décrites.

Malgré les précautions prises au cours des différentes manipulations, il nous a paru utile de vérifier que nos nouvelles souches appartenaient bien toujours à l'espèce *Rhodotorula mucilaginosa**. Toutes les souches colorées ont bien conservé les caractères essentiels qui permettent de les ranger dans l'espèce *Rhodotorula mucilaginosa*. Il n'en va pas de même pour les mutants blancs puisqu'une des caractéristiques essentielles du genre *Rhodotorula* est une coloration allant du jaune au rouge.

NAKAYAMA *et al.*[2] considèrent que la différenciation des genres *Cryptococcus* et *Rhodotorula* basée respectivement sur l'absence ou la présence de caroténoïdes est arbitraire et suggèrent que la distinction soit faite d'après la production ou non "d'amidon".

Cette manière de procéder ne lève cependant pas toute indétermination, des levures comme *Rhodotorula glutinis* variété infirmo-miniata produisant de "l'amidon".

Connaissant l'origine de nos souches blanches, il était difficile d'admettre que ces levures appartenaient à une autre espèce que *Rhodotorula mucilaginosa* bien que certains auteurs n'hésitent à annoncer la création de nouvelles espèces par simple irradiation ultraviolette[14].

Nous préférons cependant, dans notre cas, nous ranger à l'avis de B. EPHRUSSI ET Ö. WINGE et considérer qu'il s'agit bien de "mutants blancs" de *Rh. mucilaginosa*.

Modifications apportées par les mutations dans la teneur en caroténoïdes de Rh. mucilaginosa

Etude qualitative: Nous avons déterminé la nature des caroténoïdes de chaque souche à partir de plusieurs centaines de grammes de levure sèche obtenue par culture en boîtes de Roux. Chaque boîte fournit en moyenne 1 g de matière sèche.

Souche mère RMP: La solution éthéro pétrolique de pigment est passée sur colonne d'alumine désactivée, il se forme un certain nombre de zones diversement colorées dont l'élution est faite aussi sélectivement que possible. Le Tableau II indique l'aspect du chromatogramme et l'éluant utilisé pour la séparation de chaque zone.

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* Le Centraal Bureau voor Schimmelcultures a bien voulu se charger de cette vérification.

Les éluats correspondant à chaque zone sont évaporés à sec sous pression réduite, en atmosphère d'azote. Après remise en solution dans l'éther de pétrole, les fractions ainsi obtenues sont examinées au spectrophotomètre.

TABLEAU II

SÉPARATION CHROMATOGRAPHIQUE DES CAROTÉNOÏDES DE *Rh. mucilaginosa*

Les zones sont indiquées dans l'ordre croissant d'adsorption.

Zone	*Couleur*	*Eluant*
1	Incolore	Ether de pétrole
2	Jaune pâle, fluorescence bleu ciel, dans l'u.v.	Ether de pétrole + 5% éther sulfurique
3	Orange	Ether de pétrole + 10% éther sulfurique
4	Jaune pâle	Ether de pétrole + 15–20% éther sulfurique
5	Orange	Ether de pétrole + 20–25% éther sulfurique
6	Jaune pâle	Ether de pétrole + 25–50% éther sulfurique
7	Rouge foncé	Ether sulfurique pur
8	Diffuse, formée en réalité de 7 bandes peu nettes	Méthanol pur

Fraction 1: Le spectre u.v. laisse supposer qu'il s'agit d'esters de stérols; le produit d'évaporation donne une réaction de Liebermann intense: nous n'avons pas poursuivi l'étude de cette fraction.

Fraction 2: ne donne pas de spectre bien défini. Nous avons craint la présence d'esters d'acides gras aussi avons nous pratiqué une saponification à froid par la potasse méthanolique à 12%. Après extraction de l'insaponifiable par l'éther de pétrole, lavage à l'eau et séchage sur sulfate de sodium anhydre, nous avons procédé à une deuxième chromatographie sur magnésie–célite (1:1). Le développement du chromatogramme par de l'éther de pétrole contenant 2% d'acétone permet d'obtenir 2 fractions: (a) La première fraction est incolore et présente dans l'u.v. une intense fluorescence bleu-ciel. Le spectre infra-rouge montre qu'il s'agit d'une substance à longue chaîne possédant, entre autres, une seule double liaison, et une fonction cétone. Il n'y a ni noyau aromatique, ni groupe OH. L'étude de cette substance n'a pas été poursuivie. (b) La deuxième fraction est colorée en jaune pâle, le spectre montre un seul maximum à 441 mμ dans l'éther de pétrole, ce produit n'a pu être identifié, il s'agit peut être d'un produit d'altération.

Contrairement à ce que nous pensions, ces différentes fractions ne contiennent ni phytoène, ni phytofluène.

Fraction 3: Le spectre obtenu (400–424–450–475 mμ) est celui d'un mélange. Par chromatographie sur magnésie–célite (1:1) avec de l'éther de pétrole à 5% d'acétone, nous avons pu obtenir deux fractions: (a) Le pigment de la 1ère fraction a été identifié au β-carotène grâce aux caractères suivants:

– Le spectre d'absorption dans l'éther de pétrole présente des maxima situés à 424–450–477 mμ (425–450–477 mμ d'après GOODWIN[15]).

– D'autre part la chromatographie sur magnésie–célite (1:1) du mélange avec du β-carotène pur (Roche) fournit une bande unique. (b) Le pigment de la 2ème fraction interfère en partie avec le β-carotène et n'a pu être séparé de ce dernier qu'avec difficulté. Entièrement épiphasique, ce pigment semble identique au pigment X mis en évidence par H. Claes[16] dans un mutant de *Chlorella* et présente les maxima suivants: 404–426–453 mμ.

Fraction 4: Le spectre indique que la séparation avec la zone précédente n'a pas été parfaite. Par contre, sur magnésie–célite (1 : 1) avec de l'éther de pétrole à 10 % d'acétone, nous séparons facilement deux fractions: (a) La première est constituée par le pigment X. (b) La deuxième est constituée par un pigment que nous avons identifié au γ-carotène par ses caractères d'absorption et son spectre présentant les maxima suivants: 435–460–493 mμ (432–461–494 mμ d'après Goodwin[8]).

Fraction 5: Cette fraction est uniquement formée par du γ-carotène déjà caractérisé dans la fraction 4.

Fraction 6: Le comportement chromatographique et le spectre présentant des maxima situés à 415–439–469 mμ indiquent qu'il s'agit de neurosporène (415–440–470 mμ d'après Haxo[9]).

Fraction 7- Le comportement chromatographique et le spectre (458–485–518 mμ) montrent qu'il s'agit de torulène, ce pigment a été isolé par Lederer de *Rh. rubra*[10].

Fraction 8: Les pigments, trop peu abondants n'ont pu être séparés de manière satisfaisante.

Fraction 9: Le pigment présente les caractères d'un acide et semble bien, d'après nos dernières expériences, être identique à la torularhodine isolée de *Rh. rubra* par Karrer et Rutschmann[11].

Nous avons préparé l'ester méthylique de notre pigment acide par action du diazométhane; la comparaison du spectre d'absorption dans l'éther de pétrole du produit obtenu avec un échantillon authentique d'ester méthylique de la torularhodine laisse penser que notre pigment est constitué par un mélange des formes *cis* et *trans* de la torularhodine.

En fait après isomérisation par l'iode selon Zechmeister[17], l'ester méthylique de référence fournit un spectre pratiquement identique à celui que nous avons obtenu pour l'ester méthylique de notre pigment acide (voir Fig. 1).

D'ailleurs la torularhodine a été obtenue à partir de *Rh. mucilaginosa* (Hoffmann La Roche, communication personnelle).

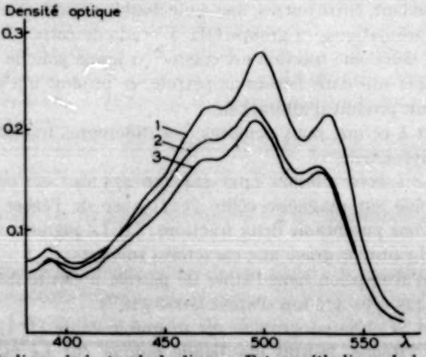


Fig. 1. 1. Ester méthylique de la torularhodine. 2. Ester méthylique de la torularhodine après isomérisation par l'iode. 3. Ester méthylique du pigment acide isolé de *Rh. mucilaginosa*.

Notre souche de *Rh. mucilaginosa* contient donc quatre pigments principaux: torularhodine, torulène, γ-carotène, β-carotène, ce qui est en accord avec les résultats de plusieurs auteurs pour d'autres espèces de *Rhodotorula* et en particulier avec ceux récents de PETERSON *et al.*[18] pour *Rh. mucilaginosa*.

Nous avons pu, d'autre part, mettre en évidence tout d'abord le neurosporène qui, en ce qui concerne les levures, n'a été signalé jusqu'à présent que par BONNER *et al.* chez *Rh. rubra* et enfin le pigment X dont c'est la première fois que l'on mentionne la présence chez une levure.

Mutant RO1: Nous avons pu caractériser quatre pigments: le torulène, γ-carotène, pigment X, β-carotène. Cette souche se distingue donc aisément de la souche mère au moins par l'absence de torularhodine. De plus, le neurosporène n'est pas synthétisé, du moins en quantité décelable, dans nos conditions expérimentales.

Mutant RB1: Malgré sa coloration blanche, nous avons pu mettre en évidence, opérant sur 200 g de levure, des traces de β-carotène. La mutation a donc provoqué, chez cette souche, une abolition pratiquement totale de la synthèse des polyènes; nous n'avons pas pu, notamment, trouver d'accumulation de phytoène ou de phytofluène, corps considérés par plusieurs auteurs comme étant les précurseurs des caroténoïdes.

Mutant RJ1: Malgré sa coloration jaune, nous avons pu caractériser chez ce mutant la torularhodine, le torulène n'a pu être décelé.

Par contre il faut noter la présence d'un caroténoïde que nous n'avons jamais rencontré dans la souche mère: le ζ-carotène. De couleur verte en solution dans l'éther de pétrole, il est adsorbé sur colonne de magnésie entre le β-et le γ-carotène.

Le spectre effectué dans l'éther de pétrole nous a fourni les valeurs suivantes: 378–399–424 mμ, NASH ET ZSCHEILE[19] donnent 378–400–425 mμ.

En outre, nous avons pu montrer chez ce mutant la présence de neurosporène, γ-carotène, β-carotène.

Mutant RJP: Ce mutant de couleur plus pâle que le précédent, contient les mêmes pigments que RJ1, y compris la torularhodine.

Mutant M1: Malgré nos nombreux essais, nous avons toujours du constater l'absence de pigment ainsi que de phytoène et phytofluène.

Mutant RJPX: A l'exception du pigment X qui n'a pas pu être caractérisé, ce mutant contient tous les pigments rencontrés dans la souche mère, c'est-à-dire: torularhodine, torulène, γ-carotène, β-carotène. En outre, de même que chez les deux autres mutants jaunes RJ1 et RJP nous avons pu caractériser le ζ-carotène. Enfin, c'est la 1ère de nos souches dans laquelle il nous a été possible de mettre en évidence le phytofluène que nous avons caractérisé grâce à son intense fluorescence bleu-vert dans l'u.v., son adsorption au-dessous du β-carotène et son spectre: 330-348-368 mμ (332–348–367 mμ d'après BONNER et al.[1])

De plus, nous avons confirmé que le polyène que nous avons isolé était bien du phytofluène par chromatographie de son mélange avec du phytofluène que nous avions isolé d'un concentré de tomates selon la technique de RABOURN *et al.*[20].

Mutant OR1: Cette souche, de coloration assez différente de celle du mutant RJPX, ne présente cependant, avec ce dernier aucune différence qualitative dans sa composition en caroténoïdes. Nous avons en effet pu caractériser: la torularhodine, le torulène, neurosporène, γ-carotène, ζ-carotène, β-carotène et phytofluène.

Mutant RJPY: L'analyse de ce mutant, dont la coloration est voisine de celle de

ORI nous a réservé une grande surprise car cinq des pigments rencontrés chez son précurseur immédiat (ORI) ont disparu et seuls le ζ-carotène et le neurosporène subsistent.

Par contre, le lycopène s'y trouve en quantité relativement élevée et nous avons pu isoler ce caroténoïde à l'état cristallisé. Il a été caractérisé par son spectre d'absorption dans l'éther de pétrole: 446–474–506 mμ (446–474–506, ESCHER[21]) et par chromatographie du mélange avec du lycopène pur (Fluka).

A notre connaissance, le lycopène n'a été mis en évidence chez les levures rouges, que dans un seul autre cas, celui d'une variété de *Rh. sanniei* étudiée par FROMAGEOT ET TCHANG[22].

Enfin, adsorbé au-dessus du lycopène, nous avons trouvé un pigment que ses caractères d'adsorption et son spectre 504–535–572 mμ dans le sulfure de carbone, 466–494–528 mμ dans l'éther de pétrole, nous ont permis de l'identifier à la *rhodoviolascine* (500–532–570 mμ) dans le sulfure de carbone et (466–494–528 mμ) dans l'éther de pétrole selon KARRER ET SOLMSSEN[23]. C'est la première fois semble-t-il que ce pigment qui constitue la majeure partie des caroténoïdes de certaines bactéries photosynthétiques[13] est mentionné chez une levure rouge.

Etude quantitative

Afin de diminuer les risques d'oxydation et d'isomérisation, nous avons travaillé sur des quantités de levure beaucoup plus faibles que précédemment (de l'ordre de quelques grammes) permettant ainsi des extractions et des chromatographies plus rapides et nous avons vérifié que la reproductibilité de nos dosages était satisfaisante.

Le Tableau III résume les résultats que nous avons obtenus dans l'étude de chaque souche, seuls les mutants RBI et MI qui ne contiennent pas de caroténoïdes n'y figurent pas.

L'examen de ce tableau montre que: (a) Le taux des caroténoïdes totaux est variable il est nettement diminué dans ROI et RJPY (*a fortiori* dans RBI et MI) par rapport à RMP alors qu'il est augmenté dans ORI. (b) Le taux de certains caroténoïdes varie considérablement. La torularhodine qui forme les 4/5 des pigments de la souche mère, n'existe plus ou ne se rencontre qu'en quantité inférieure à 5 % chez les mutants. Le β-carotène, au contraire qui représente moins de 5 % des pigments de RMP, forme plus de 60 % de ceux de ROI et RJP. Le γ-carotène passe de 1 % chez RMP à 42 % chez RJI. (c) Des caroténoïdes nouveaux peuvent apparaître comme le lycopène qui reste indécelable dans la souche mère mais constitue 85 % des caroténoïdes de RJPY.

Modifications apportées par le 2-hydroxybiphényle dans la teneur en caroténoïdes de Rh. mucilaginosa

Dans un précédent mémoire[24] nous avons montré que sous l'influence du 2-hydroxybiphényle nous obtenons, en culture, une levure pratiquement blanche. Cette dernière n'est cependant pas totalement dépourvue de caroténoïdes. En effet, nous avons extrait à partir de 50 g de levure sèche ayant poussé en présence de 2-hydroxybiphényle une solution jaune très pâle que nous avons soumise à la chromatographie. Quatre expériences ont été réalisées dans les mêmes conditions et ont donné chaque fois des résultats qualitatifs identiques. Nous avons pu caractériser le ζ-carotène, le neurosporène et le phytoène à l'exclusion de tout autre polyène. Leur dosage a été

TABLEAU III

TENEUR DE *Rh. mucilaginosa* ET DE SES MUTANTS EN POLYÈNES

Les résultats sont la moyenne d'au moins deux expériences, ils sont exprimés en µg/g, les nombres entre parenthèses indiquent, pour chaque souche, le pourcentage de chacun des pigments dosés par rapport au total.

	RMP	*RO1*	*RJ1*	*RJP*	*RJPX*	*OR1*	*RJPY*
Torularhodine	97.8 (81.27)	—	4.78 (4.85)	4.61 (3.62)	3.02 (2.98)	4.55 (3.13)	
Spirilloxanthine	—	—	—	—	—	—	1.52 (2.0)
Lycopène	—	—	—	—	—	—	65.66 (86.4)
Torulène	15.6 (12.96)	8.9 (16.51)	?	?	1.83 (1.82)	0.26 (0.18)	—
Neurosporène	—	—	5.22 (5.30)	4.79 (3.77)	5.03 (4.98)	5.1 (3.51)	3.29 (4.32)
γ-Carotène	1.36 (1.13)	8.5 (15.77)	41.43 (42.11)	35.38 (27.85)	19.37 (19.23)	46.43 (31.98)	—
ζ-Carotène	—	—	6.35 (6.45)	2.23 (1.75)	7.76 (7.68)	9.0 (6.19)	2.90 (3.82)
Pigment X	Traces	1.6 (2.97)	—	—	—	—	—
β-Carotène	5.57 (4.62)	34.88 (64.73)	40.6 (41.26)	80.3 (63.07)	54.41 (53.90)	74.93 (51.61)	—
Phytofluène	—	—	—	—	9.52 (9.43)	4.90 (3.37)	2.69 (3.52)
Total	120.33	53.88	98.38	127.31	100.94	145.17	76.05

fait dans deux expériences, en voici les résultats exprimés en μg/g de levure sèche:

Neurosporène	0.84	0.92
ζ-Carotène	1.66	1.44
Phytoène	6	10

Le phytofluène est donc totalement absent de ces cultures. Ce fait n'est pas surprenant, NAKAYAMA[25] n'a que rarement pu caractériser le phytofluène chez des levures rouges et d'autre part, BONNER *et al.* qui ont été les premiers auteurs à signaler la présence de ce polyène chez *Rh. rubra*, ont noté sur des souches conservées pendant deux ans une diminution très importante du taux de phytofluène[26].

Etude de l'apparition des pigments après élimination de 2-hydroxybiphényle

20 ml d'une culture de *Rh. mucilaginosa* sur 2-hydroxybiphényle, âgée de 24 h, servent à ensemencer 40 boîtes de Roux contenant chacune 200 ml de milieu semi-synthétique gélosé et 40 μg/ml de 2-hydroxybiphényle. La levure est récoltée au bout de 4 jours à l'aide d'une solution de 2-hydroxybiphényle à 40 μg/ml puis centrifugée à 0° et lavée 4 fois rapidement à l'eau glacée: dans ces conditions, il ne reste plus ni sucre, ni 2-hydroxybiphényle dans la dernière eau de lavage.

La masse cellulaire est placée dans 8 l d'une solution de phosphate monopotassique *M*/15. Nous prélevons alors rapidement un échantillon qui servira au dosage des caroténoïdes à l'instant zéro puis un courant d'air stérile est envoyé dans la suspension. Au bout de 20 min, le changement de teinte est déjà perceptible. Des échantillons sont prélevés au bout de 2 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h; la levure est tuée par l'acétone et les caroténoïdes extraits de la manière habituelle.

Les résultats d'une de ces expériences sont indiqués dans le Tableau IV.

TABLEAU IV

DOSAGE DES DIFFÉRENTS CAROTÉNOÏDES EN FONCTION DU TEMPS, APRÈS ÉLIMINATION DU 2-HYDROXYBIPHÉNYLE

Les résultats sont exprimés en μg/g de levure sèche.

Temps (h)	*Torularhodine*	*Torulène*	*Neurosporène*	*γ-Carotène*	*ζ-Carotène*	*β-Carotène*	*Total*
0	0	0	0.40	0	3.07	0	3.47
2	0.83	0	0.53	0	5.03	0	6.39
6	2.7	0	0.66	0	8.25	0	11.61
12	21	0	0.72	0	3.12	2.73	27.67
24	58.75	23.30	0.68	13.05	2.48	11.88	113.25
36	70.2	29.6	0.70	15.12	0.68	28.5	145.63
48	72.2	32.1	0.66	7.6	0	32.2	144.76
72	68	8.65	0	7.30	0	15.95	101.02

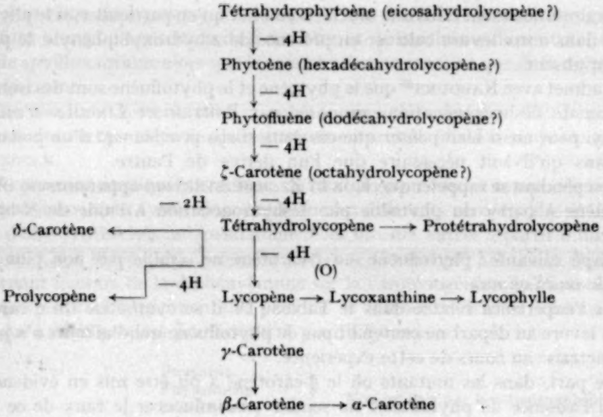
DISCUSSION

L'étude de BONNER *et al.*[1] constitue toujours, à notre connaissance, la seule tentative pour aborder le mécanisme de la caroténogénèse des levures rouges par l'analyse systématique de mutants. Les conditions dans lesquelles ont opéré les auteurs précédents sont très différentes des nôtres; en particulier les mutants isolés proviennent tous d'une seule irradiation u.v. de la souche mère alors que la plupart de nos souches sont obtenues après plusieurs irradiations successives, ce qui peut expliquer que nous enregistrons des modifications beaucoup plus marquées.

BONNER *et al.* n'ont pas mis en évidence de torularhodine et le torulène constitue dans leur souche mère 70 % des caroténoïdes totaux. Trois de leurs mutants sont restés rouges (IV, VI, VII), deux mutants (I et II) sont orangé et se distinguent de la souche mère par une très forte diminution du torulène et une augmentation sensible du γ-carotène, surtout chez le mutant II.

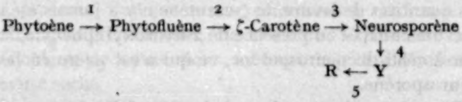
Nous constatons de même chez notre souche RJ1 une disparition pratiquement totale du torulène et une très forte augmentation du γ-carotène. Chez RO1, la disparition de la torularhodine est également accompagnée d'une augmentation sensible du β-carotène. Il semblerait donc d'après ces résultats que le γ-carotène constitue le précurseur du torulène et le β-carotène celui de la torularhodine.

De fait, la structure polyisoprénique des caroténoïdes incite à concevoir une filiation dans leur synthèse et c'est ainsi qu'à la suite d'une étude génétique très étendue sur les tomates, PORTER ET LINCOLN[27] ont proposé le schéma ci-après.


Tétrahydrophytoène (eicosahydrolycopène?)
— 4H
Phytoène (hexadécahydrolycopène?)
— 4H
Phytofluène (dodécahydrolycopène?)
— 4H
ζ-Carotène (octahydrolycopène?)
— 2H
— 4H
δ-Carotène
Tétrahydrolycopène
Protétrahydrolycopène
— 4H
(O)
— 4H
Prolycopène
Lycopène
Lycoxanthine
Lycophylle
γ-Carotène
β-Carotène
α-Carotène

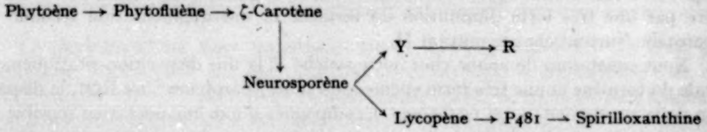
(d'après GOODWIN[15])

Certains auteurs ont pu mettre en évidence une telle filiation chez les microorganismes et tout récemment NAKAYAMA[28] a montré à l'aide de mutants de *Rhodopseudomonas sphéroïdes* que deux pigments Y et R se synthétisent selon le schéma:


1
2
3
Phytoène
Phytofluène
ζ-Carotène
Neurosporène
4
R
Y
5

La réaction 1 serait bloquée chez le mutant bleu-vert qui ne contient plus que du phytoène, la réaction 4 est bloquée chez le mutant vert qui accumule principalement du neurosporène et du dihydroxyneurosporène alors que la réaction 5 n'a pas lieu chez le mutant brun qui voit s'accumuler du neurosporène et du pigment Y.

Par une étude comparée de l'apparition des caroténoïdes dans des conditions normales et après action de la diphénylamine, JENSEN *et al.*[29] concluent également chez *Rhodospirillum rubrum*, à une filiation selon le schéma:


Phytoène → Phytofluène → ζ-Carotène
Neurosporène
Y → R
Lycopène → P481 → Spirilloxanthine

Nous allons envisager si ces schémas peuvent être appliqués aux résultats expérimentaux que nous venons de rapporter en étudiant les différentes étapes indiquées par les auteurs.

1. L'existence de la première de ces étapes: Phytoène → phytofluène semble douteuse chez *Rh. mucilaginosa* du fait que ces deux polyènes n'ont pas pu être caractérisés simultanément dans une souche donnée et qu'en particulier, si le phytoène est présent dans notre levure cultivée en présence de 2-hydroxybiphényle, le phytofluène en est absent.

Si l'on admet avec RABOURN[30] que le phytoène et le phytofluène sont des isomères, la succession de déshydrogénations proposée par PORTER ET LINCOLN n'est plus valable et on peut aussi bien penser que ces deux corps proviennent d'un précurseur commun sans qu'il soit nécessaire que l'un dérive de l'autre.

Il faut cependant se rappeler que KOE ET ZECHMEISTER[6] ont apparemment obtenu du phytofluène à partir du phytoène par déshydrogénation à l'aide de N-bromosuccinimide.

2. L'étape suivante: phytofluène → ζ-carotène ne semble pas non plus avoir lieu chez *Rh. mucilaginosa*.

D'après l'expérience relatée dans le Tableau IV il se synthétise du ζ-carotène alors que la levure au départ ne contenait pas de phytofluène qui d'ailleurs n'a jamais pu être caractérisé au cours de cette expérience.

D'autre part, dans les mutants où le ζ-carotène a pu être mis en évidence, la présence ou l'absence de phytofluène ne semble pas influencer le taux de ce caroténoïde.

Enfin d'après les travaux de NASH *et al.*[19] le ζ-carotène serait un isomère du phytofluène et non un déhydrophytofluène ce qui va également à l'encontre de la théorie des déshydrogénations successives.

3. Le passage du ζ-carotène au neurosporène est également douteux chez *Rh. mucilaginosa* car si le neurosporène a pu être caractérisé dans la souche mère en travaillant sur de fortes quantités de levure, le ζ-carotène n'y a jamais été mis en évidence. De plus dans la levure cultivée en présence de 2-hydroxybiphényle, le taux de ζ-carotène est supérieur à celui du neurosporène, ce qui n'est guère en faveur du passage ζ-carotène → neurosporène.

4. La filiation neurosporène → lycopène ne semble s'opérer ni chez la souche mère, ni chez les mutants autres que RJPY. Il ne nous a pas été possible en effet, de mettre en évidence le lycopène même en travaillant sur des quantités de levure sèche de l'ordre de 300 g. De plus dans RJPY où le lycopène représente 86 % des polyènes totaux, le neurosporène représente 4 %, c'est-à-dire un taux semblable à celui des autres mutants où il ne se forme pas de lycopène.

5. L'enchaînement lycopène ⟶ γ-carotène ⟶ β-carotène semble également peu vraisemblable. En effet nous n'avons pu déceler de lycopène dans la souche mère qui contient cependant de petites quantités de γ-carotène et β-carotène; d'autre part après élimination du 2-hydroxybiphényle le γ-carotène apparaît après le β-carotène et non avant, de plus la présence de lycopène n'a pu être notée dans ce cas là.

L'apparition de pigments n'existant pas dans la souche mère comme par exemple le lycopène, que nous trouvons à un taux élevé dans RJPY, est assez difficile à expliquer.

Cependant on connaît des mutations correspondant à "l'acquisition" d'un gène[31] et d'autres où l'apparition d'un caractère est due à la mutation d'un gène dominant qui exerçait une action inhibitrice.

Quelques cas d'apparition de pigments chez des mutants sont signalés dans la littérature: Bonner *et al.*[1] n'ont trouvé de ζ-carotène chez *Rh. rubra* que dans le mutant "brun-orangé". Saperstein *et al.*[38] étudiant les pigments de mutants de *Corynebacterium michiganense* obtenus par action du sulfate d'uranium signalent dans le mutant orangé, l'apparition de β-carotène et de canthaxanthine et dans le mutant rose, de spirilloxanthine alors que la souche mère ne contient que de la cryptoxanthine et du lycopène.

Ces résultats laissent penser, qu'au moins chez certains microorganismes, la synthèse des caroténoïdes ne s'effectue pas suivant la filiation proposée par Porter et Lincoln.

Ne pouvant appliquer de schéma de filiation pour rendre compte de nos résultats expérimentaux, nous avons alors pensé que les différents caroténoïdes étaient susceptibles d'être synthétisés indépendamment les uns des autres à partir d'un précurseur commun. C'est l'idée qu'a également soutenue Goodwin[33] à la suite de ses travaux concernant l'action de la diphénylamine sur la caroténogénèse de *Phycomyces*.

Nos résultats expérimentaux peuvent se transcrire dans le schéma ci-dessous, analogue à celui proposé par Goodwin pour *Phycomyces*.

$$\text{Précurseur commun}\left\{\begin{array}{l}\left.\begin{array}{l}\text{Phytoène}\\ \text{Phytofluène}\\ \zeta\text{-Carotène}\\ \text{Neurosporène}\end{array}\right\}\quad\text{Non inhibés par le 2-hydroxybiphényle}\\[3ex] \left.\begin{array}{l}\gamma\text{-Carotène}\\ \beta\text{-Carotène}\\ \text{Torulène}\\ \text{Torularhodine}\end{array}\right\}\quad\text{Inhibés par le 2-hydroxybiphényle}\end{array}\right.$$

Cette théorie proposée par Goodwin semble parfaitement pouvoir expliquer nos résultats. La présence d'un précurseur commun est confirmée par les données exposées dans le Tableau III. En effet, au début de l'expérience, la levure contient 3.47 μg de caroténoïdes/g de levure sèche, au bout de 36 h d'aération, nous trouvons 145.63 μg/g de levure sèche.

Il n'est guère possible d'identifier le précurseur accumulé au phytoène, puisque le taux de ce polyène est d'environ 10 μg/g de levure sèche ce qui est nettement insuffisant pour justifier un tel accroissement de la teneur en caroténoïdes.

La nature de ce précurseur reste à déterminer mais il est vraisemblable qu'il n'appartient pas à la série de Porter et Lincoln et il pourrait s'agir d'une molécule beaucoup plus petite que l'édifice en C40 proposé dans la théorie de la filiation, ainsi que l'a suggéré MACKINNEY[34] pour expliquer l'augmentation considérable de la synthèse du β-carotène par *Phycomyces* sous l'influence de la β-ionone.

D'après cet auteur, la partie centrale de la molécule se synthétiserait indépendamment de ses extrémités. Il est intéressant de constater que cette hypothèse rejoint la méthode employée par ISLER *et al.*[35] pour effectuer la synthèse des caroténoïdes en réalisant la condensation de 2 fragments symétriques avec un fragment central.

REMERCIEMENTS

Nous remercions tout particulièrement Messieurs les Professeurs B. EPHRUSSI et Ö. WINGE pour les commentaires qu'il nous ont adressés au sujet des mutations et Monsieur le Professeur GOODWIN pour l'intérêt qu'il a bien voulu prêter à notre travail.

Nos remerciements vont également à Mlle CLAES pour les renseignements qu'elle a bien voulu nous fournir au sujet du pigment X et à la firme Hoffmann-La-Roche (Bâle) pour l'envoi d'un échantillon d'ester méthylique de torularhodine et l'interprétation du spectre infrarouge.

RÉSUMÉ

1. La souche de *Rhodotorula mucilaginosa* que nous avons étudiée contient les caroténoïdes suivants, en μg/g de matière sèche:

Torularhodine 98, torulène 16, γ-carotène 1.5, β-carotène 5.5. Elle ne contient ni phytoène, ni phytofluène.

2. Par irradiations u.v. successives, nous avons pu isoler des souches stables dont la composition qualitative et quantitative est très différente de celle de la souche mère; par rapport à cette dernière, le taux de certains caroténoïdes diminue ou même devient nul, il peut aussi augmenter et il apparaît parfois des caroténoïdes n'existant pas dans la souche mère.

3. *Rh. mucilaginosa* cultivée en présence de 2-hydroxybiphényle devient blanche mais contient encore de petites quantités de polyènes.

Par lavage, l'inhibiteur peut être éliminé et une synthèse endogène des pigments peut être mise en évidence.

4. Les résultats obtenus ne paraissent pas compatibles avec l'idée d'une filiation entre les différents polyènes.

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EXCHANGE REACTIONS CATALYZED BY γ-AMINOBUTYRIC–GLUTAMIC TRANSAMINASE

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(Received October 19th, 1959)

SUMMARY

γ-Aminobutyrate-glutamate transaminase catalyzes exchange reactions between γ-aminobutyrate and succinic semialdehyde and between L-glutamate and α-ketoglutarate. Several analogs were tested for inhibition of each of the exchange reactions; for a given analog, approximately equal inhibitions were obtained in both exchange reactions. Glutarate and butyrate were the most effective analogs tested.

INTRODUCTION

The purification of a soluble γ-aminobutyric-glutamic transaminase from a strain of *Pseudomonas fluorescens* has permitted a kinetic analysis of the course of the reaction described by equation (1). The results of this work were consistent with the hypothesis that only binary complexes of the transaminase and its substrates are involved and permitted the formulation of a mechanism[1] summarized by equations (2) to (5).

$$\gamma\text{-aminobutyrate} + \alpha\text{-ketoglutarate} \rightleftharpoons \text{succinic semialdehyde} + \text{L-glutamate} \quad (1)$$

$$RCH_2NH_2 + ECHO \rightleftharpoons E' \quad (2)$$

$$E' \rightleftharpoons RCHO + ECH_2NH_2 \quad (3)$$

$$RCOCOOH + ECH_2NH_2 \rightleftharpoons E'' \quad (4)$$

$$E'' \rightleftharpoons RCH_2NH_2COOH + ECHO \quad (5)$$

The enzyme may be pictured as an aldehyde (ECHO), as an amine (ECH_2NH_2) and as two enzyme–substrate complexes (E', E'') of unspecified structure. Since pyridoxal phosphate has not been definitely identified as the prosthetic group of the transaminase it is only by analogy to other transaminases that the aldehyde and amino forms of the enzyme may be equated with the equivalent forms of the coenzyme. The symbol, R, represents $-CH_2COOH$, the radical common to all four substrates.

Implicit in this formulation is the capacity of the enzyme to catalyze an exchange of isotope between either of the substrate amino acids and their respective carbonyl analogues. Thus, incubation of the enzyme with γ-[^{14}C]aminobutyrate and succinic semialdehyde should lead to the partition of the isotope between the pair in a manner determined[2] by their respective concentrations (equations (2) and (3)). In an entirely analogous fashion an exchange reaction between L-glutamate and α-ketoglutarate (equations (4) and (5)) would be expected.

In the present report, it is demonstrated that γ-aminobutyric-glutamic transaminase catalyses isotopic exchange between γ-aminobutyrate and succinic semialdehyde as well as between glutamate and α-ketoglutarate. The inhibitory properties of several structural analogs of the substrates are examined with respect to the exchange reactions and the implications of these results for the mechanism of the overall transaminase reaction are discussed.

EXPERIMENTAL

Methods and materials

γ-[1-^{14}C]aminobutyric acid (specific activity 1 μC/μmole) and α-[1,2-^{14}C]ketoglutaric acid (0.9 μC/μmole) were obtained from Merck and Co., Ltd. of Canada. The enzyme preparation was Fraction II obtained from a strain of *Pseudomonas fluorescens* by methods described previously[1].

Succinic semialdehyde, α-ketoglutarate and γ-aminobutyrate concentrations were determined spectrophotometrically[3].

Measurements of the transamination and exchange reactions were based upon a method for the separation of the amino acid substrate by adsorption on Dowex 50 and subsequent elution with ammonia. The enzyme incubations were carried out in a total volume of approx. 0.1 ml at 30° and terminated by the addition of 0.1 ml of 0.1 *M* sodium bisulfite. The samples were transferred quantitatively to a water-washed column of 200–400 mesh Dowex 50 X-8 (H^+ form) consisting of 1 ml of wet resin in a 1-cm diameter, coarse, sintered-glass funnel. After addition of the incubation mixture, the columns were eluted with 7 ml of distilled water; the eluates were collected in graduated test tubes. When the radioactivity of the amino acid was to be determined, a second elution with 7.0 ml of 10 % NH_4OH (concentrated ammonium hydroxide diluted ten-fold) was performed. Eluate volumes were recorded and 100-µl aliquots were added to 10 ml of liquid scintillation phosphor solution. The phosphor solution contained 0.3 % 2,5-diphenyloxazole in toluene–methanol (3:1, v/v). A Packard Model 314 liquid scintillation counter was used for ^{14}C determinations.

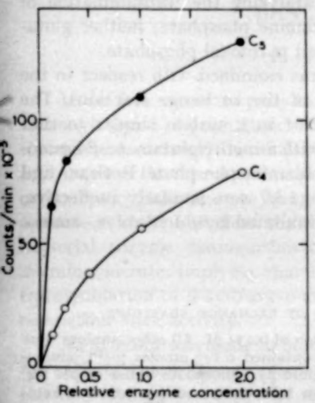

Fig. 1.

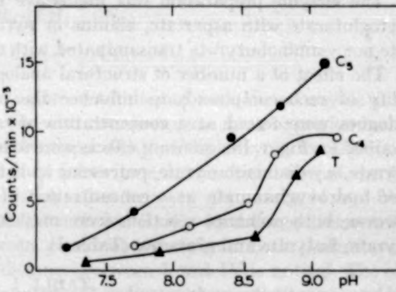

Fig. 2.

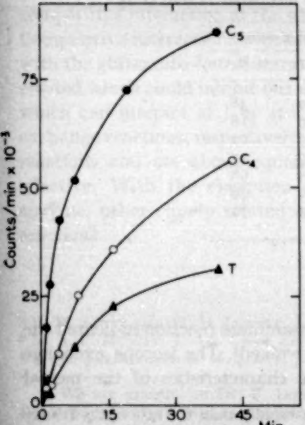

Fig. 3.

Fig. 1. Isotope exchange as a function of enzyme concentration. (C_4) Each tube contained 0.126 µmole of γ-aminobutyrate, 1.35 µmoles of succinic semialdehyde, 5 µmoles of sodium pyrophosphate buffer, pH 8.4, and 10 µl of enzyme in a final volume of 105 µl. (C_5) Each tube contained 0.24 µmole of α-ketoglutarate, 1.25 µmoles of glutamate, 5 µmoles of sodium pyrophosphate buffer at pH 8.4 and 10 µl of enzyme in a final volume of 100 µl. In C_4, the radioactivity was initially in the γ-aminobutyrate and the curve represents the accumulation of radioactivity in the water eluates, *i.e.*, in succinic semialdehyde. In C_5, the radioactivity was initially in the α-ketoglutarate and the curve represents the accumulation of radioactivity in the ammonia eluates, *i.e.*, in glutamate. The value of 1.0 on the abscissa corresponds to the amount of enzyme used in the experiments reported in Table I. Incubation was for 12 min at 25°.

Fig. 2. Effect of pH on exchange reactions. (C_4) Each tube contained 10 µmoles of pyrophosphate buffer, 0.25 µmole of γ-[^{14}C]aminobutyrate, 5 µmoles of succinic semialdehyde, and 1.6 µl of enzyme in a total volume of 115 µl. (C_5) Each tube contained 10 µmoles of pyrophosphate buffer, 0.5 µmole of α-[^{14}C]ketoglutarate, 1.25 µmoles of glutamate, and 1.6 µl of enzyme in a total volume of 110 µl. Incubation was for 10 min at 25°. The pH of each sample was measured on an aliquot of the incubation mixture.

Fig. 3. Time course of the exchange reactions and of transamination. Each incubation mixture contained 50 µmoles of potassium pyrophosphate at pH 8.4 and 40 µl of enzyme in a total volume of 0.79 ml. Flask C_4 (○) contained, in addition, 1.26 µmoles of γ-[1-^{14}C]aminobutyrate and 13.5 µmoles of succinic semialdehyde. Flask C_5 (●) contained 3.0 µmoles of α-[1,2-^{14}C]ketoglutarate and 12.5 µmoles of L-glutamate. Flask T (▲) contained 3.0 µmoles of α-[1,2-^{14}C]ketoglutarate and 12.5 µmoles of γ-aminobutyrate. Each point represents the radioactivity found in that substrate which was non-radioactive initially, a 50-µl aliquot being withdrawn at the indicated time. Incubation was at 25°.

The relative rates of the two exchange reactions as functions of enzyme concentration, pH and incubation time are shown in Figs. 1, 2 and 3, respectively. The γ-aminobutyrate-succinic semialdehyde exchange (C_4 exchange reaction) proceeds more slowly than the glutamate-α-ketoglutarate exchange (C_5 exchange reaction). In Fig. 3, the initial rate of the over-all transamination reaction may be noted to approximate the initial rate of the C_4 exchange reaction[*].

The enzyme preparation was ineffective in catalyzing the transamination of α-ketoglutarate with aspartate, alanine or pyridoxamine phosphate; neither glutamate nor γ-aminobutyrate transaminated with added pyridoxal phosphate.

The effect of a number of structural analogs was examined with respect to the ability of such compounds to influence the rate of the exchange reactions. The analogues were tested at a concentration of 0.016 *M* in a system similar to that described for Fig. 1. Insignificant effects were found with α-methylglutamate, β-aminobutyrate, α,γ-diaminobutyrate, putrescine and ethanolamine phosphate. Both DL- and allo-β-hydroxyglutamate at a concentration of 0.032 *M* were similarly ineffective. However, both exchange reactions were markedly inhibited by β-hydroxy-γ-aminobutyrate, butyrate and glutarate (Table I).

TABLE I

EFFECT OF STRUCTURAL ANALOGS ON VELOCITY OF EXCHANGE REACTIONS

β-hydroxyglutamate (DL, allo) was present at a concentration of 0.032 *M*. All other analogs were present at a concentration of 0.016 *M*. (C_4) Each tube contained 0.126 μmoles γ-[^{14}C]aminobutyrate, 1.35 μmoles succinic semialdehyde, 5 μmoles sodium pyrophosphate buffer at pH 8.4, and 5 μl of enzyme in a final volume of 100 μl. (C_5) Each tube contained 0.30 μmole α-ketoglutarate, 1.25 μmoles glutamate, 5 μmoles sodium pyrophosphate buffer at pH 8.4, and 5 μl enzyme in a final volume of 100 μl. Samples were incubated 12 min at 25°.

Analog	*% of control*	
	C_4	C_5
DL-β-hydroxy-γ-aminobutyrate	50	38
β-aminobutyrate	99	118
Butyrate	37	62
Glutarate	37	47
Succinate	85	98
α-methyl glutamate	90	78
β-hydroxy glutamate	93	74

DISCUSSION

Previous analysis of the γ-aminobutyric-glutamic transaminase reaction indicated the binary nature of the enzyme-substrate complexes involved[1]. The isotope exchange technique employed here permitted a study of the characteristics of the partial reactions, *i.e.*, those involving interaction of the enzyme with either the four-carbon or the five-carbon substrate pair.

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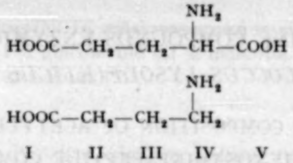
[*] In Fig. 3 the rate of reaction T is seen to decrease more rapidly than the rate of C_4. It may be noted that the equilibrium constant for C_4 and C_5 is 1 while that for reaction T is 0.1 and thus less favorable to the accumulation of succinic semialdehyde. Moreover, the initial rates of C_4, C_5 and T cannot be expected to become identical even if more adequate methods were available to measure them. The steady state equations describing the initial velocities may be shown to contain different constants in each case. Not only are rate constants combined in T from C_4 and C_5, but a substrate inhibition of α-ketoglutarate on T has been demonstrated[1]. Although the data seem to support the rate limiting role of the initial reaction between γ-aminobutyrate and enzyme, the rate constants of one or more intermediate complexes may be of significance.



$$\mathrm{HOOC{-}CH_2{-}CH_2{-}\overset{NH_2}{\overset{|}{C}}H{-}COOH}$$

$$\mathrm{HOOC{-}CH_2{-}CH_2{-}\overset{NH_2}{\overset{|}{C}}H_2}$$

If the two substrate pairs can be assumed to interact with the coenzyme at the carbonyl–amine position, their interaction with the apoenzyme must be predominantly through the carboxyl functions. The stereospecificity of the enzyme for L-glutamic acid requires that the α-carboxyl (V) must participate in the orientation of the five-carbon pair. Since the enzyme also distinguishes between glutamic acid and other α-amino acids, it is evident that the distal carboxyl (I) is a further requirement. The bacterial enzyme distinguishes between γ-aminobutyrate and both β-alanine and Δ-aminovalerate, implying that the distance between I and IV is critical. The slow transamination of β-hydroxy-γ-aminobutyrate* indicates that substitution at III is compatible with activity.

An attempt was made to inquire into the question of the number of sites involved for each of the exchange reactions since it is evident that the common carboxyl function (I) might occupy the same or a different locus on the enzyme surface for the two substrate pairs. If the interaction of the substrates differs only at V, any competitive interaction at the site for I should interfere with both exchange reactions. Competitive interactions which involved only V would be expected to interfere largely with the glutamate–ketoglutarate exchange. In no case (Table I) was a selective action elicited which could inhibit one exchange reaction and not the other. Carboxylic acids which can interact at I or at I and V might be expected to inhibit the C_4 and C_5 exchange reactions, respectively. In fact, butyrate and glutarate inhibit both exchange reactions and are about equally effective. In contrast, succinate is relatively ineffective. With the exception of β-hydroxy-γ-aminobutyrate, a substrate of the enzyme, other closely related compounds had only minor effects on the exchange reactions.

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* We are grateful to Dr. F. Irreverre for informing us that the transaminase can utilize β-hydroxy-γ-butyric acid as a substrate.

Biochim. Biophys. Acta, 40 (1960) 457–461

ACETYLHEXOSAMINE COMPOUNDS ENZYMICALLY RELEASED FROM *MICROCOCCUS LYSODEIKTICUS* CELL WALLS

I. ISOLATION AND COMPOSITION OF ACETYLHEXOSAMINE AND ACETYLHEXOSAMINE-PEPTIDE COMPLEXES

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(Received August 29th, 1959)

SUMMARY

Some of the dialyzable products of the digestion of *Micrococcus lysodeikticus* cell walls by lysozyme and by a similar enzyme secreted by a *Streptomyces* have been isolated and their compositions determined. The two simplest substances released by both enzymes are (a) a di-saccharide of N-acetylmuramic acid-N-acetylglucosamine and (b) an N-acetylmuramic acid-N-acetylglucosamine complex. Seven peptide-acetylamino sugar complexes have been isolated. All seven compounds contain lysine, glutamic acid, glycine, alanine in the same molecular proportions as found in the original cell wall and a di-saccharide moiety of N-acetylmuramic acid and N-acetylglucosamine. One of the peptide-amino sugar complexes contains in addition a polysaccharide moiety [N-acetylmuramic acid-N-acetylglucosamine]$_{10}$.

INTRODUCTION

As reported earlier[1], the dialyzable fraction of lysozyme-digested *Micrococcus lysodeikticus* cell walls, contains at least one "small fragment" distinguishable by its R_F value and by its positive MORGAN AND ELSON reaction. It was also shown that this "small fragment", possibly di-saccharide, is composed of N-acetylglucosamine and an unknown N-acetylamino sugar, subsequently shown to be muramic acid[2,3]. Muramic acid was first detected in hydrolysates of spore peptides[4,5] and later found in many bacterial cell walls[6–9]. The structure of 3-O-α-carboxethyl-D-glucosamine has been proposed for it by STRANGE AND KENT[10].

This paper describes the isolation and the composition of several compounds giving a positive MORGAN AND ELSON reaction, which are released from *Micrococcus lysodeikticus* cell walls during digestion with egg-white lysozyme and with the F_1 fraction obtained from a bacteriolytic *Streptomyces* culture[11–13].

Abbreviations: AG = N-acetylglucosamine; DMAB = *p*-dimethylaminobenzaldehyde.

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MATERIALS AND METHODS

Cell walls preparations

Micrococcus lysodeikticus (NCTC 2665) was grown in vigorously aerated nutrient broth supplemented with 1 % glucose and 0.1 % mannitol. The cell walls were made by the method of SALTON AND HORNE[14].

Enzymes

Crystalline egg-white lysozyme (Armour Laboratories) was used throughout these studies. The *Streptomyces* bacteriolytic enzyme was isolated by one of us (J.M.G.) from culture filtrates (280 L) by a selective adsorption[13] at pH 7 on an Amberlite IRC 50 column. The resin was then washed three times with a *N* KCl solution, suspended in a buffer 0.08 *M* KOH–0.08 *M* boric acid–0.42 *M* NaCl and treated with 100 ml (per 200 g of resin) of a 4 *N* KOH–*N* KCl solution. The supernatant, dialyzed and lyophilized (2.1 g) was readsorbed on the minimum of Amberlite IRC 50 XE 64, previously equilibrated against a 0.0125 *M* veronal buffer pH 7, and eluted at the same pH by a 0.05 *M* veronal–0.2 *M* NaCl buffer. This elution was carried out four times by stirring the resin with the selected buffer system. The extracts were dialyzed together against distilled water and the white insoluble material (0.195 g) was collected. This last step of purification by precipitation at zero ionic strength was repeated three times and finally the insoluble purified enzyme (0.086 g) was dissolved in the buffer system KH_2PO_4–K_2HPO_4, μ 0.01–pH 7, 0.1 *M* NaCl (2 mg of dry weight enzyme/ml of solution), divided in lots of 1 ml each and freeze-dried. This final "F_1 preparation", at a concentration of 10 µg/ml, is without any detectable activity upon heated *Escherichia coli* cells, casein and keratin[11,13] and reduces the turbidity of a staphylococcal cell suspension[13,14] (lab. strain SA_3) to 50 % of its original value in 1 h.

Preparation of the dialyzable fractions of enzyme-digested walls

1 g of cell walls (dry wt.) was incubated for 20 h at 37° in the presence of 1 mg enzyme (lysozyme or F_1 preparation) in 40 ml 0.05 *M* ammonium acetate. The clear solution was dialyzed against 150 ml distilled water at 4° for 24 h. Dialysis was repeated three times and the dialyzable fraction freeze-dried *in vacuo* over H_2SO_4. Under these conditions, the dialyzable fraction accounted for approx. 35 % of the original weight of cell walls after F_1 treatment, and approximately 45 %, after lysozyme treatment.

Chromatography

Chromatography was performed on Whatman No. 1 paper in one dimension (descending in *n*-butanol–acetic acid–water (3 : 1 : 1, v/v/v)) or in two dimensions at right angles (ascending in pyridine–water (4 : 1, v/v) and descending in the butanol–acetic acid solvent).

Electrorheophoresis

Electrorheophoresis was carried out without avoiding the evaporation of the solvent from the papers by supporting them on a frame 34 cm/20 cm. Whatman 3MM paper and glass fiber filter paper (No 934-AH; Hurlbut Paper Co.) were used under a constant voltage of 700 V with the following buffer systems: 2 N acetic acid (pH 2.35); glacial acetic acid–pyridine–water (2:4:1000) (pH 5.5); 0.02 N acetic acid + NH_3 (d 0.88) to pH 8.9. The material to be separated by electrorheophoresis on 3MM paper was deposited either at the equilibrium position near the center of the sheet (when performed at pH 2.35), or close to the positive electrode (for pH 5.5) or to the negative electrode (for pH 8.9). For all the experiments on glass paper, the substances were deposited at the center of the sheet.

Sprays and color reactions

On the chromatograms and electrorheophoreograms, the amino acids and amino sugars were detected with ninhydrin (0.2 % in acetone) and estimated by extracting the spots in 5 ml of acetone–water (3:1, v/v) and measuring the O.D. of the solution at 570 mμ. The reducing substances were detected with ammoniacal silver nitrate reagent and the acetylamino sugar-type substances by SALTON's method[15].

Hexosamines in solution were determined by a modification[16] of the method of ELSON AND MORGAN.

The technique used by REISSIG *et al.*[17] for the MORGAN-ELSON acetylhexosamine reaction was applied to the purified acetylamino sugar complexes as follows: 1 ml of sample, in 0.05 M $Na_2B_4O_7$, was heated for an adequate period of time (see EXPERIMENTAL) in a boiling water bath, and, after cooling to room temperature, mixed with 2.5 ml of EHRLICH's reagent (1 g DMAB [p-dimethylaminobenzaldehyde] in 100 ml glacial acetic acid containing 2 ml of conc. HCl). After 20 min at 37°, the O.D. was read at 585 mμ.

EXPERIMENTAL

Paper chromatographic properties

After digestion by lysozyme or by F_1, the dialyzable material corresponding to 5 mg of original *Micrococcus lysodeikticus* cell walls was examined by paper chromatography (Fig. 1).

The chromatograms obtained from both enzymic digests showed the presence

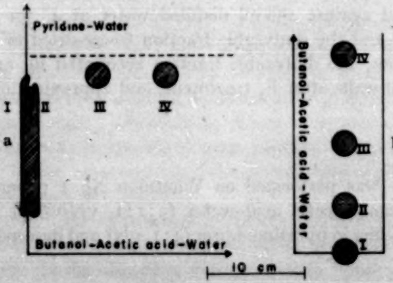

Fig. 1. Diagram of paper chromatograms of the MORGAN-ELSON positive substances released from *Micrococcus lysodeikticus* cell walls by lysozyme and F_1 digestion. (a) Two-dimensional chromatography for 12 h in pyridine–water (ascending) and 18 h in butanol–acetic acid–water (descending). (b) One-dimensional chromatography for 30 h in butanol–acetic acid–water (descending). IV and III components: DMAB and $AgNO_3$ positive; ninhydrin negative. II and I components: DMAB, $AgNO_3$ and ninhydrin positive.

of four spots detected by the modified MORGAN-ELSON spray reaction[15]. The components were called respectively LI, LII, LIII, LIV (after lysozyme digestion) or F_1I, F_1II, F_1III, F_1IV (after F_1 digestion), the index I to IV indicating an increasing migration in the butanol–acetic acid solvent. In pyridine–water, components III and IV migrated together and were very close to the solvent front, while components I and II showed considerable tailing.

Chromatograms were also sprayed with ammoniacal silver nitrate and ninhydrin reagents. Compounds I, II, III and IV showed a positive reaction with silver nitrate, but only compounds I and II were ninhydrin positive. Additional silver nitrate positive and ninhydrin positive spots could be detected but were not further examined*.

Electrorheophoretic properties

For the studies of the electrorheophoretic properties of the four MORGAN-ELSON positive substances liberated by lysozyme and F_1 from *Micrococcus lysodeikticus* cell walls, the dialyzable, digested fractions were applied to Whatman No. 1 paper as a band (400 µg/cm) and irrigated with the butanol–acetic acid system. After the four spots were located by spraying a narrow strip with the modified MORGAN-ELSON spray reagent[15], the bands were eluted with water and the solution evaporated to dryness.

After electrorheophoresis on Whatman 3MM paper under the conditions described in MATERIALS AND METHODS, the substances were located by their positive reactions with silver nitrate and DMAB reagents (components III and IV) and, in addition, with ninhydrin (components I and II).

The migrations of the main substances are illustrated in Fig. 2. This figure also shows the behavior of a neutral substance (N-acetylglucosamine).

At pH 5.50 and 8.90, all the substances were found to have a negative charge with a migration decreasing in the following order: LIII and F_1III; LIV and F_1IV; LI and F_1I; LII and F_1II. At pH 2.35, LIII and F_1III, and LIV and F_1IV were neutral while LI and F_1I, and LII and F_1II behaved as basic substances, the former pair being less positively charged than the latter pair.

These electrorheophoretic properties and color reactions strongly suggested that the components LIII and F_1III, LIV and F_1IV, were two different types of N-acetylamino sugar complexes, with free carboxylic groups belonging to muramic acid. On the other hand, the behavior of the components LI and F_1I, LII and F_1II showed that they were probably peptide-N-acetylamino sugar complexes.

The electrorheophoretic properties of these complexes were also examined on glass fiber paper with the same buffer systems. These studies were undertaken with fractions already purified by chromatography in butanol–acetic acid and electrorheophoresis on Whatman 3MM paper. Since SALTON's modified MORGAN-ELSON spray reagent gave irregular and generally rather weak reactions on this glass paper, the substances were detected only with the ninhydrin and silver nitrate reagents. The substances migrated more readily on the glass paper than on Whatman paper. Therefore, the time of electrorheophoresis on glass paper was less and the various substances could not be applied close to the electrode to which they were attracted by their electrical charges (*e.g.*, applied at pH 5.5 near the positive electrode, components III and IV migrate off the glass paper).

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* It is worth noting that the silver nitrate reagent showed a fifth well-separated spot, ninhydrin and MORGAN-ELSON negative, with a R_F 0.95 in pyridine–water and 0.50 in butanol–acetic acid. It originated from the dialysis tubing and appeared to be glycerol or a related substance.

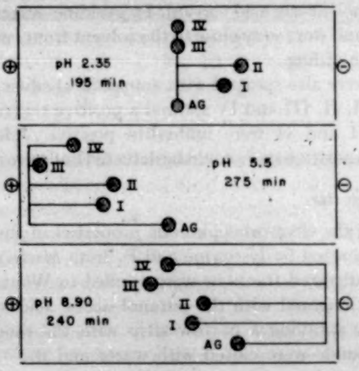

Fig. 2. Diagram of electrorheophoretic behavior on Whatman 3MM paper of the MORGAN-ELSON positive substances released from *Micrococcus lysodeikticus* cell walls by lysozyme and F_1 digestion. IV and III components: DMAB and $AgNO_3$ positive; ninhydrin negative. II and I components: DMAB, $AgNO_3$ and ninhydrin positive. AG: N-acetylglucosamine.

At pH 5.5 and after 1 h of electrorheophoresis, components III and IV migrated 12 cm from the center of the sheet towards the positive electrode. Although no further separation of these components occurred under these conditions, this electrorheophoresis was a useful step in the purification of both compounds because of the removal of one or more contaminating reducing substances (with a migration of 3 to 15 cm).

The situation was the same for the components I and II. Contaminating substances were removed by further electrorheophoresis on glass fiber at pH 2.35 (2 h) or at pH 5.5 (1 h). In addition, a further separation was achieved with the F_1II fraction. This fraction, separated after a 5-cm migration on Whatman 3MM at pH 2.35, was resolved by electrorheophoresis on glass fiber, at the same pH (2 h), into four components with a migration from the center of the sheet of 0; 4.5; 7 and 13 cm. Only two intermediate fractions F_1II B and F_1II C were both silver nitrate and ninhydrin positive.

Separation and purification

The technique was based on the chromatographic and electrorheophoretic properties described above. In every case, the dialyzable, digested fractions were first separated by chromatography in the butanol–acetic acid system and the four MORGAN-ELSON positive areas eluted and taken to dryness.

Fractions III and IV were successively submitted to electrorheophoresis at pH 5.5, first on 3MM paper (deposited near the positive electrode) and afterwards on glass fiber paper, the transfer of the substances from one paper to the other being made by applying the band of 3MM paper to the center of the glass fiber paper. The substances were finally eluted from the glass paper, filtered through a sintered glass filter and evaporated to dryness by lyophilization.

The purification of fractions I and II was more complicated since, unlike fractions III and IV, each of them contained several compounds, with different electrorheophoretic properties. Furthermore, the number and behavior of the compounds in fractions I and II varied in different enzymic digests. These variations perhaps suggest some difference in the degree of the enzymic digestion of the preparations or in the efficiency of the dialysis. Whatever the explanation, the purifications were carried out in the manner reported above for the components III and IV, by successive electrorheophoresis at pH 2.35 and 5.5 on Whatman 3MM paper and on glass fiber paper.

Composition of the purified compounds LIII, F_1III, LIV and F_1IV

Preliminary experiments showed that these purified complexes consisted only of N-acetylglucosamine and N-acetylmuramic acid, with no free amino groups. Estimations of the two hexosamines liberated by acidic hydrolysis and of the purity of the complexes III and IV were performed as indicated below. 800 μg of each compound were hydrolyzed with 1 ml 2 *N* HCl, 2 h, 100° and each hydrolysate divided into four aliquots called series A, B, C, D and treated as follows.

(A) Aliquots were chromatographed in two dimensions and the ninhydrin positive spots, corresponding to glucosamine and muramic acid, were eluted with the acetone–water solvent and the O.D. determined at 570 mμ. (B) Aliquots were chromatographed in the butanol–acetic acid solvent and the areas corresponding to glucosamine and muramic acid were eluted, lyophilized and analyzed[16]. (C) The same procedure as (B) was carried out but the analyses were performed on cut-out areas of the paper placed directly in test tubes. In this case, the proportion H_2O–acetylacetone buffer was 2/1.5 ml. (D) The ELSON-MORGAN reaction[16] was directly performed on $NaHCO_3$ neutralized hydrolysates. For these hexosamine analyses, standard N-acetylglucosamine was also hydrolyzed and treated as above.

Table I shows the O.D. of glucosamine/O.D. of muramic acid obtained with ninhydrin (series (A)) and the ELSON-MORGAN tests (series (B), (C), (D)). In series (D), muramic acid concentrations could be calculated since the amount of glucosamine was known by the above procedures ((A), (B), (C)).

TABLE I

RATIO GLUCOSAMINE/MURAMIC ACID, IN TERMS OF OPTICAL DENSITY, OF THE HYDROLYZED AMINO SUGAR COMPLEXES RELEASED FROM *Micrococcus lysodeikticus* CELL WALLS BY LYSOZYME AND F_1 DIGESTION

Fraction	*Ratio estimated by*			
	Ninhydrin	ELSON-MORGAN *Reaction*		
	Series (A)	*Series (B)*	*Series (C)*	*Series (D)*
F_1III	1.13	2.40	3.28	2.30
L III	1.02	2.30	2.98	2.50
F_1IV	1	2.26	3.20	2.35
L IV	1.03	2.25	3.03	2.42
Average	1.04	2.30	3.14	2.39

Both ninhydrin and Elson-Morgan ratios showed that the same proportion of glucosamine and muramic acid occurred in the four amino sugar complexes. By ultracentrifugation (1.5 %; ammonium acetate buffer 0.5 M; 238,300 × g), amino sugar LIV gave an S value of 0.3 S which indicated that it was a di-saccharide (P.M. about 500). Therefore, a 1 to 1 proportion of N-acetylglucosamine to N-acetylmuramic acid was assigned to this compound as well as to the three other amino sugar complexes.

The Elson-Morgan ratio varied from 2.30 to 3.14 according to the technique. However, it is known[5] that muramic acid cannot be estimated with reference to glucosamine unless the conversion factor is known for the method used. Strange and Kent[10] reported that the ratio glucosamine/muramic acid, expressed in terms of Elson-Morgan reaction coloration, was respectively 1.78, 1.85 and 1.56 when the estimation was made by the methods of Elson and Morgan[18], Dishe and Borenfreund[19] and Tracey[20]. The method of Neuhauer *et al.*[16] was selected for the present studies because it minimized interferences by sugars and amino acids, but the effectiveness of the extraction procedure for the different chromogens is not known.

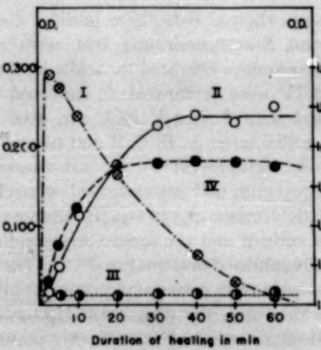

Fig. 3. Effect of duration of heating of the borate solution on color development, at 585 mμ, in Morgan-Elson reaction of the amino sugar complexes IV and III and of the amino sugar-peptide complexe LII, released from *Micrococcus lysodeikticus* by enzymic digestion. Right ordinate: I; N-acetylglucosamine 100 μg; left ordinate: II: F$_1$IV 75 μg (corrected weight); III: F$_1$III 75 μg (corrected weight); IV: LII B (preparation 4b) 200 μg.

The purity of each amino sugar complex was estimated by referring to a standard glucosamine curve. It was 75 % for F$_1$III, LIII and F$_1$IV and 90 % for LIV.

Acetylhexosamine Morgan-Elson *reaction*: This reaction was applied to the disaccharides LIV and F$_1$IV using the modified method of Reissig *et al.*[17]. As shown in Fig. 3, heating the alkaline solution for 3 min provided a maximum coloration for the N-acetylglucosamine while the reaction was only slightly positive for the disaccharides. Heating for a longer time resulted in an increased coloration until a maximum was finally reached after 40 min. The absorption spectrum of the colored complexes was identical to that given by N-acetylglucosamine or by the "small fragment" previously isolated[1] from *Micrococcus lysodeikticus* cell walls, with two peaks at 545 and 585 mμ and a minimum at 565 mμ.

By this technique, the di-saccharides were differentiated from the N-acetylglucosamine and from the amino sugar complexes III which as shown in Fig. 3 gave no appreciable color (a faint color was, however, obtained after heating 1 h at 120°).

Fig. 4 shows the relationship between the O.D. and the concentration of di-saccharides. As the proportionality coefficient was exactly the same for both LIV and F_1IV, we used this property for their estimation. On the basis of a molecular weight of 497, the molar extinction coefficient was found to be 4870 at 585 mμ.

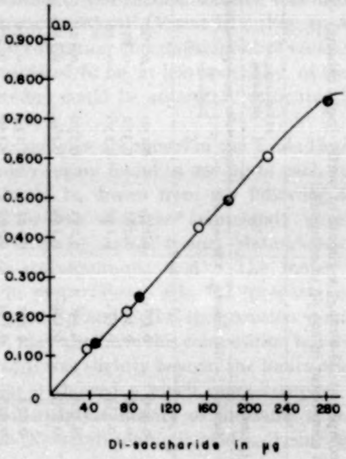

Fig. 4. Relationship between the O.D. (585 mμ) and the amount of di-saccharides LIV (○) and F_1IV (●) released from *Micrococcus lysodeikticus* by enzymic digestion, using the MORGAN-ELSON reaction. The weight of the amino sugar complexes are corrected on the basis of a purity of 75 % for F_1IV and 90 % for LIV. Calculation of the molar extinction coefficient: $\frac{497 \cdot 10^6 \times 3.5 \times 0.0028}{1000} = 4870$.
P.M. of the di-saccharide, 497; volume of the solution, 3.5 ml; angular coefficient, 0.0028.

Composition of the purified compounds F_1I, F_1II, LI and LII

Seven amino sugar-peptide complexes were isolated from three different preparations (called 3, 4a and 4b) of the dialyzable fractions: F_1I, F_1II B and F_1II C (preparation 3), LI, LII (preparation 4a) and F_1I, F_1II (preparation 4b). As had been found by STRANGE AND POWELL[4] with the *Bacillus* spore peptides, complete hydrolysis of the different compounds I and II demanded fairly strong conditions, so that the hydrolyses were carried out in 6 *N* HCl, for 20 h at 100°.

600 μg of each compound, as well as synthetic control mixtures of equal quantities of lysine, glutamic acid, glycine, alanine and N-acetylglucosamine, were hydrolyzed and divided into three aliquots. These aliquots were further treated exactly in the same way as described above for the series (A) (two-dimensional chromatography, detection with ninhydrin and estimation of amino acids and amino sugars at 570 mμ), C (one-dimensional chromatography; ELSON-MORGAN reaction on the separated

TABLE II

COMPOSITION OF *Micrococcus lysodeikticus* CELL WALLS AND OF THE PURIFIED AMINO SUGAR-PEPTIDE COMPLEXES RELEASED BY ENZYMIC DIGESTION

Results expressed in µmoles of each component in 200 µg of compound. The rough estimate of purity (right column) was obtained by adding the weight of each component in 200 µg of compound.

No. dialyzable fraction	Complexes	*Ninhydrin estimation*						ELSON-MORGAN *estimation*		MORGAN-ELSON *estimation*	*Purity*
								Glucosamine and muramic acid			
		Lysine	*Glutamic acid*	*Glycine*	*Alanine*	*Glucosamine*	*Muramic acid*	*Test series* *C*	*D*	*Di-saccharide*	
3	F_1I	0.170	0.132	0.140	0.406	0.156	0.120	0.150	0.160	0.122	80%
	F_1II B	0.034	0.030	0.030	0.106	0.348	0.282	0.352	0.355	0.026	89%
	F_1II C	0.206	0.200	0.116	0.534	0.174	0.152	0.136	0.150	0.154	92%
4a	F_1I	0.147	0.144	0.158	0.361	0.136	0.153	0.130	0.127	0.133	78%
	F_1II	0.155	0.144	0.174	0.363	0.149	0.149	0.163	0.157	0.160	88%
4b	L I	0.130	0.125	0.145	0.241	0.101	0.101	0.124	0.144	0.085	77%
	L II	0.220	0.182	0.166	0.545	0.164	0.197	0.158	0.210	0.180	106%
Intact cell walls		0.176	0.146	0.133	0.436	0.132	0.157				

glucosamine and muramic acid) and D (Elson-Morgan reaction on the neutralized hydrolysates).

The content of glucosamine and amino acids in each compound was obtained with reference to glucosamine and amino acids controls. The content of muramic acid was calculated from the O.D. of the colored solution on the basis of a ratio O.D. glucosamine/O.D. muramic acid equal to 1 in series (A) and to 3.14 and 2.39 in series (C) and (D).

Moreover, each unhydrolyzed compound gave a positive Morgan-Elson reaction when the duration of heating of the alkaline solution was increased to 40 min as had been the case for the di-saccharides F$_1$IV and LIV (Fig. 3). Also, there was a direct relationship between the coloration and the amount of compound so that the amino sugar moieties were considered to be, at least partially, of the same type as the free di-saccharides and therefore could be accurately estimated by applying the molar extinction coefficient of 4870.

The results of these analyses are shown in the Table II and expressed in µmoles of amino acids and amino sugars found in 200 µg of each compound. Although no structural inferences should be drawn from the following formula, the molecular compositions, at least for five of these compounds were identical within the limits of experimental error (± 20 %): lysine$_1$–glutamic acid$_1$–glycine$_1$–alanine$_{2-3}$–[N-acetylglucosamine-N-acetylmuramic acid]$_1$. The molar proportion of alanine varied with the different preparations: 2 for LI (preparation 4b); 2.50 for F$_1$I and F$_1$II (preparation 4a); 3 for F$_1$I and F$_1$II C (preparation 3) and LII (preparation 4b).

LI (preparation 4b) may also have this composition but its relative molar proportion of di-saccharide (0.70) was slightly beyond the limits of error.

F$_1$II B (preparation 3) showed a much more complex amino sugar structure bonded to a di-saccharide-peptide moiety of the same type as the preceding one: [(N-acetyl)-glucosamine-(N-acetyl)-muramic acid]$_{10}$-[lysine$_1$-glutamic acid$_1$-glycine$_1$-alanine$_3$]-[di-saccharide].

The µmoles of various amino acids and amino sugars estimated by the elution of ninhydrin-stained spots from the hydrolysate of 200 µg of intact *Micrococcus lysodeikticus* cell walls are also given in Table II. It should be noted that the molar proportion of amino acids and amino sugars found in the original cell walls was that found in the amino sugar-peptide complexes isolated from the enzymic digests.

CONCLUSIONS

Among the small fragments released from the walls during their digestion with F$_1$ or lysozyme, two types of amino sugar complexes [N-acetylglucosamineN-acetylmuramic acid]$_n$ were isolated: a di-saccharide (n = 1) previously detected by Salton[1] and an oligo-saccharide with n unknown. They could be differentiated on the basis of the positive Morgan-Elson acetylhexosamine reaction (with a molar extinction coefficient of 4870) given, in solution, by the di-saccharide. It should be recalled however that a positive Morgan-Elson reaction is given by the oligosaccharide when the test is done on paper by Salton's technique.

Several amino sugar-peptide complexes were also isolated. Their peptide moieties were always of the same type: lysine$_1$–glutamic acid$_1$–glycine$_1$–alanine$_{2-3}$. It is noteworthy that the same proportion of amino acids was found in the original cell walls. These results suggest that the wall polymer is made up of peptide sub-units of the same type, although there is as yet no information on the actual sequences of amino acids in such peptides. On the other hand, their amino sugar moieties usually consisted of the di-saccharide itself, and, in one case, of an additional residue consisting of ten molecules of glucosamine and ten of muramic acid, both very probably acetylated.

It is now possible to give some idea of the relative quantities of the various dialyzable complexes which are formed as a result of the enzymic digestion of the cell walls. After a lysozyme-digestion of 100 mg of *Micrococcus lysodeikticus* cell walls, it was found that the dialyzable fraction contained 7.2 mg of free di-saccharide, 10.2 mg of oligo-saccharide and 3.2 mg of bond di-saccharide. This latter figure would correspond to 8 mg of peptide-di-saccharide complexes. Thus the substances which have been investigated represent at least 25 % of the weight of the original cell walls and 50 % of the dialyzable material released during their enzymic digestion.

ACKNOWLEDGEMENTS

We should like to thank Professor R. Y. Stanier for his kind hospitality. We also wish to thank Miss M. Lannes for her excellent assistance. For one of us (J.M.G.), this research was performed during an appointment supported by the International Cooperation Administration under the Visiting Research Scientists Program administered by the National Academy of Sciences of the United States of America.

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ACETYLHEXOSAMINE COMPOUNDS ENZYMICALLY RELEASED FROM *MICROCOCCUS LYSODEIKTICUS* CELL WALLS

II. ENZYMIC SENSITIVITY OF PURIFIED ACETYLHEXOSAMINE AND ACETYLHEXOSAMINE-PEPTIDE COMPLEXES

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(Received August 29th, 1959)

SUMMARY

Free di-saccharide (N-acetylglucosamine-N-acetylmuramic acid) is released from a purified amino sugar complex, probably tetra-saccharide, by the action of egg-white lysozyme and of a similar enzyme secreted by a *Streptomyces*. The di-saccharide is also released from a purified poly-acetylamino sugar-peptide-di-saccharide compound by the action of the same enzymes on its poly-acetylamino sugar moiety. Differences in the affinity of egg-white lysozyme and of the *Streptomyces* enzyme for their substrates are discussed.

A second bacteriolytic enzyme, also secreted by the *Streptomyces*, liberates free disaccharide from the purified peptide-di-saccharide and poly-acetylamino sugar-peptide-di-saccharide complexes by splitting the bond between the carboxyl group of muramic acid and the amino group of the peptide moiety.

INTRODUCTION

Preliminary experiments showed that the various purified acetyl-hexosamine compounds[1] isolated from the dialysable fractions obtained after lysozyme- or F_1-digestion of *Micrococcus lysodeikticus* cell-walls still contained some enzymically sensitive linkages. The present paper presents the results of an investigation of the action of egg-white lysozyme and of the F_1 and F_2B fractions obtained from bacteriolytic *Streptomyces* culture, on these isolated substrates of low molecular weight.

MATERIALS AND METHODS

Conditions of chromatography and electrorheophoresis, and sprays and color reactions used, are described in the preceding paper[1].

The F_2B *Streptomyces* enzyme was isolated by adsorption at pH 5.5 on an amberlite IRC 50 resin column from 280 L of *Streptomyces* culture filtrate. This

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Abbreviations: AG = N-acetylglucosamine; AMA = N-acetylmuramic acid.

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filtrate had already been adsorbed on a column of the same resin at pH 7 in order to prepare the F_1 enzymic fraction[1]. The resin was then washed three times with a *N* KCl solution and treated with 50 ml (per 200 g of resin) of a 4 *N* KOH–*N* KCl solution. The dried product obtained by lyophilization of the dialyzed supernatant was dissolved in 500 ml of 0.1 *M* ammonium sulfate solution and precipitated by salting-out with 1500 ml of saturated ammonium sulfate solution. The insoluble brown material was resuspended in water, dialysed, taken to dryness (1.84 g) and then resuspended in phosphate buffer pH 7, μ 0.05. The supernatant, dialysed and lyophilized (1.38 g), constituted the F_2B enzyme fraction, while the material insoluble in the phosphate buffer (crude F_2A enzymic fraction) was further purified for other purposes. F_2B is entirely water-soluble.

Substrates

The substrates studied were all isolated[1] from digests of cell walls of *Micrococcus lysodeikticus* with either lysozyme or *Streptomyces* enzyme F_1. They fall into four classes: di-saccharide AG–AMA (DS); oligo-saccharide $(AG–AMA)_n$ (OS); di-saccharide-peptide (DSP): and di-saccharide-peptide-poly-saccharide [(AG–AMA)-peptide-AG_{10}-AMA_{10}] (DSPPS). When the origin of a particular substrate is relevant, it will be indicated by the suffix L (prepared after lysozyme digestion) or F_1 (prepared after F_1 enzyme digestion).

Incubation

Each purified small molecular weight compound was incubated under the same conditions: 200 μg of compound + 20 μg of enzyme (lysozyme, F_1 or F_2B) in a final volume of 0.02 ml and an ionic strength (ammonium acetate) of 0.05 (if incubated with F_1 or lysozyme) or of 0.005 (if incubated with F_2B). Incubation for 20 h at 37°.

EXPERIMENTAL

Enzymic degradation of di- and oligo-saccharides

Table I summarizes the changes produced in di- and oligo-saccharides as a result of incubation with lysozyme, F_1 and F_2B. The reaction mixtures after enzymic digestion were chromatographed and the spots were detected with SALTON's reagent[2] and ammoniacal silver nitrate.

The di-saccharide was completely resistant to F_2B, F_1 and lysozyme. The oligo-saccharide was completely resistant to F_2B, slightly sensitive to F_1 and highly sensitive to lysozyme. In the two latter reaction mixtures, only the di-saccharide and a certain amount of undigested oligo-saccharide could be detected. The oligo-saccharide must therefore be a polymer of the di-saccharide. Evidence given elsewhere[3] shows that it is a tetra-saccharide: AG–AMA–AG–AMA.

A quantitative estimation of the di-saccharide released by the actions of F_1 and lysozyme on the tetra-saccharide was made using the MORGAN-ELSON reaction (with the molar extinction coefficient 4870)[1], either directly on the digest itself or on the di-saccharide separated from the undigested tetra-saccharide by chromatography in butanol–acetic acid or by electrorheophoresis at pH 5.5 on Whatman 3MM paper. The same results were obtained estimating the hexosamines liberated from the isolated di-saccharide by 2 *N* HCl hydrolysis (2 h, 100°). The data in Table II show

TABLE I

EFFECT OF F_1, F_2B AND LYSOZYME ON THE PURIFIED AMINO SUGAR AND AMINO SUGAR-PEPTIDE COMPLEXES RELEASED FROM *Micrococcus lysodeikticus* CELL-WALLS BY A PRIMARY DIGESTION BY F_1 AND LYSOZYME

Substrates		*Substances released under the action of*		
		Lysozyme	F_1	F_2B
Di-saccharide (L or F_1)		o	o	o
Tetra-saccharide (L or F_1)		Disaccharide	Disaccharide (trace)	o
*DSP compounds**				
Name	*Preparation*			
F_1I	3	o	o	Disaccharide
F_1IIC	3	(Acetylmuramic acid)	(Acetylmuramic acid)	Disaccharide
F_1I	4a	o	o	Disaccharide
F_1II	4a	o	o	Disaccharide
L I	4b	o	o	Disaccharide
L II	4b	o	o	Disaccharide
*DSPPS compound**				
Name	*Preparation*			
F_1IIB	3	Disaccharide	Disaccharide (trace)	Disaccharide

* See ref. 1.

TABLE II

ESTIMATION OF THE DI-SACCHARIDE RELEASED, UNDER THE ACTION OF F_1, F_2B AND LYSOZYME, FROM THE PURIFIED AMINO SUGAR AND AMINO SUGAR-PEPTIDE COMPLEXES

This estimation is given in percentages calculated by dividing the [number of μmoles of disaccharide liberated × 100] by the number of μmoles of tetra-saccharide used or by the number of μmoles of di-saccharide bound to the peptide moiety and corresponding to the amount of amino sugar-peptide complex incubated.

Substrate		*Enzyme*	*Percentage of di-saccharide released, estimated by*			
			MORGAN-ELSON *reaction*			ELSON-MORGAN *reaction on the hexosamines isolated from the HCl hydrolysed di-saccharide*
			On the digest itself	*On the di-saccharide separated by*		
				Chromatography	*Electrorheophoresis*	
Tetra-*saccharide*						
(L)		Lysozyme	65	64	62	59
		F_1	11	5	5	6
(F_1)		Lysozyme	54	54	58	57
		F_1	12	8	7	12
*DSP compounds**						
Name	*Preparation*					
F_1II	4a	F_2B		78	75	
L I	4b			61	63	
L II	4b			64	63	
*DSPPS compound**						
Name	*Preparation*					
F_1IIB	3	F_2B		66	61	

* See ref. 1.

the differences in sensitivity of the tetra-saccharide towards lysozyme (54 to 65 % digestion) and towards F_1 (5 to 12 % digestion).

Action on the amino sugar-peptide complexes

1. Enzyme F_2B released 67 to 78 % of di-saccharide when incubated with DSP complexes as shown both by greater sensitivity to the ninhydrin reagent and by an increase of the positive charges of the peptide residues. Before the enzymic treatment, compound LII B (preparation 4b; Table II)[1] had a migration of 8.5 cm at pH 5.5 (700 V; 270 min; point of application: close to the positive electrode). After F_2B digestion, the electrorheophoresis showed, besides a certain amount of undigested LII B, the presence of the di-saccharide with a migration of 3 cm (*i.e.*, more negatively charged than the original compound) and the presence of the peptide residue with a migration of 14.5 cm (more positively charged). F_1 and lysozyme did not act on DSP complexes since no free di-saccharide was detected. However, with the compound F_1II C (preparation 3; Table II)[1], the butanol–acetic acid chromatograms of the F_1- and lysozyme-digests contained a small amount of a fast moving substance, ninhydrin negative and silver nitrate and *p*-dimethylaminobenzaldehyde positive, which could be free N-acetylmuramic acid.

2. The three enzymic systems, lysozyme, F_1 and F_2B liberated a certain amount of di-saccharide from DSPPS. The ninhydrin reactivity of the residual material was unchanged after incubation with F_1 and lysozyme. Coupled with the fact that these two enzymes liberate no di-saccharide from DSP compounds, this indicates that their site of attack on DSPPS must reside in the poly-saccharide $(AGA–AMA)_{10}$ residue. The residual material after treatment of DSPPS with enzyme F_2B showed an increased ninhydrin reactivity. Moreover, the amount of di-saccharide liberated represented 66 % of the di-saccharide moiety present in the untreated compound which is closely similar to the amount of di-saccharide liberated by the same enzyme from DSP complexes.

Therefore, it seems probable that F_2B released the intact di-saccharide bonded to the peptide moiety in the original compounds while the di-saccharide liberated by lysozyme and, to a less extent by F_1, originated from the poly-saccharide residue by breakage of glycosidic bonds.

Differences between egg-white lysozyme and F_1

Despite the similarities between lysozyme and F_1, they can be differentiated as follows:

Thermal denaturation: At a concentration of 2 µg/ml, unheated F_1 and lysozyme reduced the turbidity of a suspension of *Micrococcus lysodeikticus* cell walls incubated under standard conditions[4] to 50 % of its original value after 15 and 5 min of incubation respectively (Fig. 1). The activity of F_1 at a concentration of 10 µg/ml was completely destroyed by heating for 6 min in boiling water bath. With the same thermal exposure, a much more dilute solution of lysozyme (0.1 µg/ml) showed no loss of activity.

Wall acetylation: Various techniques[5] are now available for changing the lysozyme resistance of bacteria and, in the case of *Micrococcus lysodeikticus*, BRUMFITT *et al.*[6] have shown that a greater lysozyme resistance is associated with an increase in the O-acetylated groups of the walls. Complete O- and N-acetylation of the walls entirely suppressed their sensitivity to lysozyme and very strongly inhibited their sensitivity to F_1. However, by subsequent treatment with 0.1 *N* NaOH for 1 h at 37°, which partially removed the O-acetyl groups, the walls recovered a high lysozyme sensitivity (Fig. 1), while they remained very resistant to F_1: with the latter, a turbidity reduction of 50 % occurred only after 20 h of incubation in presence of 2 μg F_1/ml.

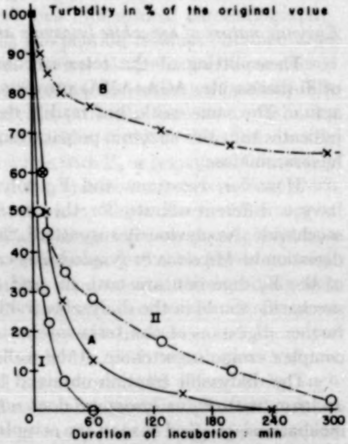


Fig. 1. Lytic action of lysozyme (I and II) and F_1 (A and B) on intact cell walls (I and A) and partially O-acetylated (and completely N-acetylated) cell walls (II and B) of *Micrococcus lysodeikticus*. 2 μg enzyme/ml: 37°; pH 7; μ 0.05; phosphates.

Comparative sensitivity of cells and walls. The sensitivity of *Micrococcus lysodeikticus* cell walls towards F_1 and towards lysozyme was of the same order of magnitude (see above). Conversely, the sensitivity of the intact cells towards the two enzymes was very different. Thus, lysozyme (2 μg/ml) caused a 50 % turbidity reduction in a cell suspension after 5 min of incubation. At the same concentration, F_1 required at least 150 min to produce a similar reduction.

Influence of the culture medium. The composition of the medium in which *Micrococcus lysodeikticus* is grown can also preferentially affect the sensitivity of the isolated cell walls to F_1. The same strain NCTC 2665 was incubated, with shaking, under the same conditions, in five different media, each of them containing 0.5 % of yeast extract, supplemented as follows: medium 1: Bacto-peptone 5 %, NaCl 1 %; medium 2: casamino acids 2 %, NaCl 1 %; medium 3: Trypticase Soy Broth 3 %, glucose 2 %; medium 4: casamino acids 2 %, glucose 1 %; medium 5: casamino acids 2 %, fructose 1 %. In each case, the walls isolated from the harvested cells showed an equal sensitivity to lysozyme (see above) whereas the walls obtained from media 1, 2, 3 were three times more sensitive to F_1 than those obtained from media 4 and 5. It must also be pointed out that the *Micrococcus lysodeikticus* cell walls used throughout these studies, had the same sensitivity towards lysozyme as has been previously found[4], but were more sensitive to the present F_1 preparation than would have been expected from the experiments recorded two years ago[4]. At that time a 50 % turbidity reduction was obtained after 120 min of incubation in presence of 10 μg F_1/ml; this compared with 15 min for a similar reduction in the presence of 2 μg/ml of the present F_1

preparation. A sample of the first enzymic preparation which was stored for two years in dry state, exhibited the same activity on the present preparation of cell walls as the present F_1 preparation. Therefore, the observed variations must be due to some differences in the nature of the two batches of *Micrococcus lysodeikticus* cell walls.

DISCUSSION

Enzymic nature of egg-white lysozyme and F_1

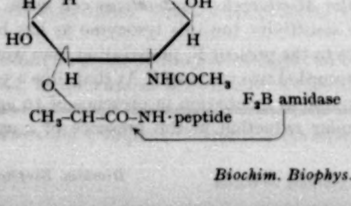
The splitting of the tetra-saccharide (AGA–AMA–AGA–AMA) into two moles of di-saccharide (AGA–AMA) proves that lysozyme has an N-acetylhexosaminidase action. The same weak, but readily detectable, action of F_1 on the tetra-saccharide indicates that this enzymic preparation contains an egg-white lysozyme-like N-acetyhexosaminidase.

However, lysozyme and F_1 polysaccharidase are two different proteins and have a different affinity for the sensitive glycosidic bond contained in the tetra-saccharide. As previously suggested[7], the di-saccharide is the end-product of lysozyme digestion of *Micrococcus lysodeikticus* cell walls. On the other hand, the end-products of the F_1 digestion are both di- and tetra-saccharides. In this latter case, the di-saccharide found in the dialysable fraction is not formed to any significant extent by further digestion of the tetra-saccharide, but rather by the destruction of a more complex amino sugar chain of the walls.

The dialysable fraction obtained by digestion of cell walls of *Micrococcus lysodeikticus* with F_1 or lysozyme does not contain any free di- or tetra-saccharides if incubation is stopped as soon as a complete clarification of the suspensions has occurred. The first action in the dissolution of the walls is therefore concerned with the breakage of glycosidic bond liberating large soluble fragments from which the di- and tetra-saccharides are subsequently split off.

Enzymic nature of F_2B

One of the key linkages in the construction of the bacterial cell wall is postulated to be an amidic linkage between the carboxyl group of muramic acid and an amino group belonging to a peptide chain. This hypothesis was strengthened by the discovery that the transfer of the peptides and muramic acid into the macromolecular structure of the walls is mediated by uridine diphosphate acetylmuramic acid nucleotides to which is linked either an alanine residue or a small peptide of glycine, glutamic acid and alanine[8–10]. It can be concluded from the experiments performed with the small purified DSP compounds that F_2B splits the linkage between the peptide and di-saccharide residues. As this action increases the positive charges of the peptide residues, it seems very probable that F_2B is an amidase acting on the linkage:



The F_2B action on intact *Micrococcus lysodeikticus* cell walls was also examined. In terms of turbidity reduction, F_2B was certainly less active than lysozyme or F_1. A concentration of 20 μg F_2B/ml reduced the turbidity of a wall suspension to 50 % of its original value after 150 min. The same turbidity reduction occurred after 5 and 15 min with 2 μg of lysozyme and of F_1, respectively. The dialysed F_2B-digested fraction contained some strongly ninhydrin positive materials which were resolved into several small peptides and free alanine by two dimensional chromatography. These peptides have the same characteristics as the peptide residues obtained after F_2B action on the purified small DSP complexes. The free alanine must be considered as an N-terminal alanine group (previously detected in the intact cell walls[11]). The dialysable fraction after digestion with F_2B enzyme, probably does not contain any poly-saccharide material, for no di- or tetra-saccharides could be detected directly or after a further incubation of this dialysable fraction with F_1 or lysozyme. Conversely the non-dialysable material was rich in MORGAN-ELSON positive substances from which lysozyme and F_1 released both di- and tetra-saccharides. These observations suggest that in the intact walls, no fragments as simple as a di- or tetra-saccharide exist in a lateral or terminal position to a peptide chain or between two such peptide chains. If the walls have first been treated by F_1, a further incubation of the non-dialysable material with F_2B enzyme releases some free di- and tetra-saccharides. At the same time, the reactivity of the whole mixture to ninhydrin increases, and by paper chromatography, the formation of a number of ninhydrin positive substances of low molecular weight can be shown. The di- and tetra-saccharides liberated by such treatment with F_2B enzyme must be regarded as small lateral or terminal branches linked to peptide chains which are held together in the intact walls by a complex poly-saccharide bridge susceptible to destruction by F_1.

Streptomyces *bacteriolytic enzymes*

The bacteriolytic activities of *Streptomyces* sp. are due to numerous different exo-enzymic systems. The existence of a lysozyme-like enzyme[12–15] was previously suspected because of the great similarity between the lytic spectrum of some *Streptomyces* culture filtrates and that of egg-white lysozyme. The N-acetyl-hexosaminidase activity of the F_1 preparation confirms this hypothesis but shows, however, that F_1 and egg-white lysozyme are two different proteins, each with a particular enzymic affinity. Other lysozymes produced by *Sarcina* sp.[16] and *Bacillus* sp.[17, 18] are known, but there is insufficient information in the literature to conclude whether they are identical to the egg-white lysozyme or to the polysaccharidase F_1.

Streptomycetes in addition secrete one other bacteriolytic poly-saccharidase, isolated by MCCARTY[19] and characterized by a lytic spectrum quite different from that of lysozyme or of F_1. MCCARTY's enzyme indeed is inactive on *Micrococcus lysodeikticus* but very active on streptococcal cell walls. On these latter walls, F_1 carried out only a slow and incomplete digestion, reducing the turbidity of a suspension of a trypsin-treated group A streptococcal cell walls to 17 % of its original value after 20 h of incubation in the presence of 20 μg enzyme/ml. It is likely that MCCARTY's enzyme is different from F_1 and lysozyme not only in its affinity but also in its specificity. We have also shown that some *Streptomyces* cultures contain at least two enzymes producing lysis of pneumococci[20] and another enzyme[21] producing lysis of streptococci

—which may be identical to McCarty's poly-saccharidase. As yet, nothing is known about their mode of action.

The experiments carried out with the F_2B fraction in the present studies strongly suggest the existence of a *Streptomyces* amidase acting on the muramic acid-peptide linkage. It was previously observed[4] that the digestion of various gram positive cell walls by *Streptomyces* bacteriolytic preparations, obtained by a similar technique to that used for the preparation of F_1 and F_2B, was accompanied by a release of small peptides and free alanine and/or glycine and that at least two different enzymic systems, called for this reason actinolysopeptidases I and II[22], were involved in these phenomena. Of course, the F_2B amidase could explain the liberation of these ninhydrin positive materials, but F_2B is inactive on *Bacillus megaterium* (K-M) and on *Staphylococcus aureus* cell walls. Moreover, F_2B seems unable to release the poly-saccharide structure from the purified small compound DSPPS. It is therefore possible to conceive the F_2B enzyme as an amidase with a very strict specificity controlled by the structure of the neighbouring poly-saccharide and peptide chains. On the other hand, besides its polysaccharidase activity on *Micrococcus lysodeikticus* cell walls, F_1 actually releases from *Bacillus* sp. and *Staphylococcus* sp. cell walls some free alanine and small peptides. In these circumstances, the F_1 preparation must be heterogeneous and must contain, in addition to the poly-saccharide, one or several "peptidases", in the broadest sense, specifically different from the F_2B amidase.

ACKNOWLEDGEMENTS

We should like to thank Professor R. Y. Stanier for his kind hospitality and Dr. J. A. Hayashi (Biological Chemistry Department, University of Illinois, Chicago) for a very generous gift of type 14, S23, group A *Streptococcus* cell walls. We also wish to thank Miss M. Lannes for her excellent assistance. This research was performed during an appointment supported by the International Cooperation Administration under the Visiting Research Scientists Program administrated by the National Academy of Sciences of the United States of America.

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THE RELATION OF METALS AND -SH GROUPS TO THE ACTIVITY OF PANCREATIC LIPASE

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(Received September 12th, 1959)

SUMMARY

A study of the effect of metal chelating agents on purified pancreatic lipase has shown that the enzyme possesses an essential stabilising metal which is probably calcium. This may be replaced less effectively by some other metals such as Mg^{++} or Sr^{++}. The effect of several divalent metal ions on the rate of triglyceride hydrolysis by the enzyme has been investigated. These metals can be divided into three groups depending as to whether they (a) increase, (b) do not affect, or (c) inhibit, the rate of hydrolysis. The toxicity of certain heavy metals has been found to be reversed by addition of amino acids.

Lipase has been found to be inhibited by certain -SH reagents, but to be quite resistant to others. It is considered that the enzyme possesses -SH groups which are not part of the active centre.

INTRODUCTION

For many years it has been known that calcium salts have a significant effect on the activity of pancreatic lipase, but the precise mechanism of the activation still remains obscure.

The activation of lipase by calcium was observed by WILLSTATTER AND MEMMEN in 1924[1]. Subsequently BAMANN AND LAEVERENZ[2] confirmed that calcium stimulated the rate of lipase action and suggested that the main function of calcium was to remove as insoluble calcium soaps the fatty acids formed in the hydrolysis. This mechanism of the action of calcium was also supported by SCHONHEYDER AND VOLQUARTZ[3].

DESNUELLE, NAUDET AND CONSTANTIN[4] found, however, that the addition of Ca^{++} in increasing concentration could alter the end products of triolein hydrolysis by lipase. In the absence of Ca^{++}, diolein was the main end product, but the addition of Ca^{++} led to an increased production of monolein. These authors considered that Ca^{++} had a dual function, a direct activation of the enzyme and an activation effect resulting from precipitation of interfacial fatty acids, thus removing the products of hydrolysis.

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[*] This work was carried out at the Biochemistry Department, University of Vermont College of Medicine, Burlington, Vt. (U.S.A.).

In contradiction to Ca^{++} activation, several heavy metals such as Cu^{++}, Hg^{++}, Co^{++} and Fe^{+++} have been found to be toxic to lipase[5].

The observation that lipase was inhibited by bromo- and iodo-acetate[5], *p*-chloromercuribenzoate and by certain arsenical drugs led to the suggestion[6] that lipase possessed –SH groups and SCOTT[7] proposed a theory of the mechanism of lipase action which was based on the assumption that sulfhydryl groups were present in the active centre.

In view of the fact that nearly all the experiments so far described, have been performed using relatively crude pancreatic extracts, it appeared desirable to investigate the role of calcium and -SH groups in the purified enzyme. This work has been undertaken using a purified lipase prepared by a method recently described by WILLS[8].

It has been found that an important function of Ca^{++} is to stabilise the active configuration of lipase and that although it is likely that –SH groups are contained in the enzyme they are unlikely to be components of the active centre.

A preliminary account of this work has already been described[9].

EXPERIMENTAL

Materials

Lipase was purified by magnesium sulphate precipitation and dialysis as previously described[8]. The resulting sodium chloride solution of the enzyme was diluted 1000–2000 times with water for use. For most experiments a solution containing 2γ protein was used.

Glycerides were obtained from Messrs. Kodak Ltd. and were used as purchased.

Methods

Five different methods were used for the determination of lipase activity and, whenever possible, results obtained with one method were checked using another: (a) The manometric method was used for most experiments with a sodium bicarbonate–carbon dioxide buffer system. The manometers were gassed either with N_2–CO_2 (95:5) or with 100 % CO_2, the concentration of bicarbonate being adjusted to give a resulting pH of 7.4. Details of this method have previously been described[10]. (b) A continuous titration method previously described[10]. (c) A continuous titration method in which the pH was measured by the glass electrode and kept constant by the continuous addition of 0.01 *N* NaOH. In a typical experiment 18 ml H_2O and 1 ml of tributyrin emulsion in gum arabic were brought to pH 7.5 and 1 ml enzyme solution added. The rate of addition of alkali was measured for a convenient time period. (d) The method of BALLS, MATLACK AND TUCKER was used in a slightly modified form as previously described[11]. (e) The method of FIORE AND NORD[12] was used for a few experiments.

The contents of the reaction flasks were shaken at 100 times/min in a water bath at 37° when methods (a), (d) and (e) were used, whilst the reaction mixtures used in methods (b) and (c) were stirred with a magnetic stirrer.

Tripropionin or tributyrin were the most commonly employed substrates, either either untreated or emulsified in 0.3 % gelatin or in 10 % gum arabic[13]. In addition, for comparison purposes, mono- and di-propionin, mono- and di-butyrin, trihexanoin, trioctanoin, trilaurin, tripalmitin and triolein were used in certain experiments.

RESULTS

The effect of metal complexing agents on lipase—the metal constituent of lipase

Dialysis of purified lipase against de-ionised water for 48 h did not cause any significant reduction in activity.

Lipase was, however, found to be strongly inhibited by the addition of a metal complexing agent such as Versene, which when used in a concentration of $2 \cdot 10^{-3}\,M$, caused 70% inhibition after contact with the enzyme for 15 min at 37°. The inhibiting effect of Versene was, however, found to be markedly dependent on the temperature of the mixture and the time that Versene had been in contact with the lipase. In order to investigate these factors more extensively, the enzyme was incubated in borate buffer at pH 8.0 with Versene ($2 \cdot 10^{-3}\,M$) for various time periods and at various temperatures between 5° and 50°. At the end of the period the temperature was rapidly adjusted to 20° and an excess of Ca^{++} added to combine with all the added Versene. Enzyme determinations were carried out at 37°. At low temperatures, *e.g.* at 5° and with short contact periods Versene had no effect on lipase, but the enzyme was found to be much less stable to heat in the presence of Versene (Table I).

TABLE I

EFFECT OF VERSENE* ON THE HEAT STABILITY OF LIPASE

Temp.		*Enzyme activity (Activity of untreated enzyme = 100)*	
		After 10 min exposure	*After 14 h exposure*
5°	Control	100	100
	Versene	100	60
20°	Control	100	83
	Versene	100	40
35°	Control	91	57
	Versene	65	9
50°	Control	64	0
	Versene	14	0

* Concentration, $2 \cdot 10^{-3}\,M$.

The addition of Ca^{++} ($10^{-2}\,M$) completely protected the lipase if added before addition of the Versene, but, once inactivation had occurred, neither Ca^{++} nor any other substance tested could restore the activity.

This lack of stability of the lipase to heat in presence of Versene appeared to be due to the removal of an essential stabilising metal. In order to ascertain which metals were able to function as stabilisers, lipase was treated in borate buffer pH 8.0, with Versene ($2 \cdot 10^{-5}\,M$) for 15 min at 5°. This treatment removes the metal but allows the enzyme to retain full activity. A solution ($10^{-2}\,M$) of the metal ion under investigation was then added and left with the lipase for 5 min at 5°. The concentration of metal ion added was more than adequate to remove the Versene by chelation, so that the excess metal ion was available for combination with the lipase. After the elapse of the 5-min period the lipase so treated was exposed at 50° for 10 min. It was then cooled to 37° and its enzymic activity compared (a) with the activity of the untreated enzyme exposed to 50° and (b) with the activity of the enzyme exposed in the presence of Versene at 50°. Any metal that enabled an activity similar to that of (a) to be obtained may be considered as a possible normal metallic constituent of lipase, whereas, if the enzymic activity was found to be similar to that of (b), then any such metal is unlikely to be a normal lipase constituent.

Calcium was found to be the most effective metal in stabilising lipase after Versene treatment but several other metals were partially effective (Table II).

TABLE II

A COMPARISON OF THE EFFECT OF DIVALENT METAL IONS ON THE RESTORATION OF LIPASE ACTIVITY AFTER TREATMENT OF THE ENZYME WITH VERSENE

After treatment with Versene ($2 \cdot 10^{-3}$ *M*), lipase was exposed to 50° for 10 min in presence of a 10^{-2} *M* solution of the metal ion. Enzymic activity was determined at 37°.

System	*Rate of tributyrin hydrolysis*
Control	100
Versene	20
Versene + Ca^{++}	88
Versene + Sr^{++}	83
Versene + Mg^{++}	80
Versene + Ba^{++}	62
Versene + Mn^{++}	56
Versene + Co^{++}	12

In the experiments so far described, the effect of Versene on lipase was examined at pH 8.0 in borate buffer. These experiments were extended to examine the effect of Versene when the pH was varied between 4.0 and 9.0. A buffer of the required pH was added to the lipase solution, followed by Versene ($2 \cdot 10^{-3}$ *M*). The mixture was exposed to 50° for 10 min, when it was rapidly cooled, brought to pH 7.4 and Ca^{++} (10^{-2} *M*) was added to combine with the Versene. Enzyme determinations were carried

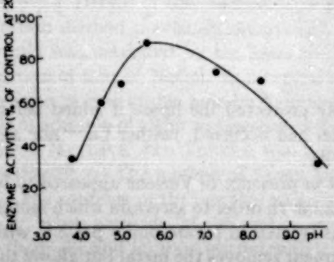

Fig. 1. The effect of pH on the heat stability of lipase. The enzyme was exposed to 50° for 10 min at the specified pH, cooled and adjusted to pH 7.4 for activity determinations. A comparison was then made with the activity of the enzyme at a comparable pH kept at 20°.

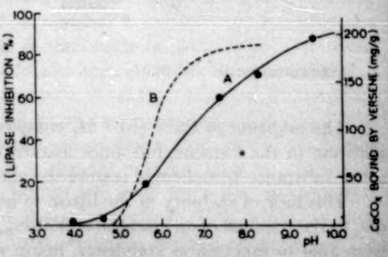

Fig. 2. The effect of pH on Versene inactivation of lipase. Curve A—Lipase inactivation by versene as a function of pH. Enzyme exposed to 50° for 10 min in presence of $2 \cdot 10^{-3}$ *M* versene at specified pH. Curve B—Binding of Ca^{++} by Versene as a function of pH (details from MARTELL AND CALVIN[14]).

out at pH 7.4. Lipase was found to be most stable to heat at about pH 5.6 (Fig. 1). The effect of Versene was markedly pH dependent, being very small when the solution was more acid than pH 5.0, but the inactivation effect increased with increasing pH. In Fig. 2 a comparison is made of the effect of pH on Versene inactivation of lipase with the effect of pH on the binding of Ca^{++} by Versene. A close similarity can be observed.

In addition to Versene several other metal complexing agents were tested on lipase. The procedure followed in each case was exactly similar to that using versene, the complexing agent under test being added to lipase in borate buffer at pH 8.0, the mixture exposed to 50° for 10 min, cooled and the enzyme activity measured. No substance tested was as effective as Versene, but the percentage reduction of enzyme activity was found to be closely paralleled by the increase of the stability constant for the calcium complex as shown in Table III.

TABLE III

COMPARISON OF THE EFFECTS OF SUCCINATE, ASPARTATE, OXALATE, CITRATE AND VERSENE ON THE HEAT STABILITY OF LIPASE

Enzyme exposed for 10 min at pH 8.0 to 50° after treatment with specified reagent.

Addition	*Log of the stability constant[14] for Ca^{++}*	*Concn. ($M \times 10^3$)*	*Enzyme activity (Control = 100 at 20°)*
None	—	—	75
Succinate	1.2	3.3	75
Aspartate	1.6	3.3	75
Oxalate	3.0	1.0	58
Citrate	3.2	1.0	41
Versene	10.77	0.2	14

The effect of metal ions on lipase action

Although it is well known that the addition of Ca^{++} speeds up the rate of hydrolysis of glycerides of long chain fatty acids[1,2], it was found to have a much less, but still a significant effect on the rate of hydrolysis of the glycerides of short chain fatty acids such as tripropionin or tributyrin. Thus, using the method of BALLS, MATLACK AND TUCKER, the rate of hydrolysis of un-emulsified triolein was found to be increased 4.4 times by Ca^{++} (0.01 M), whereas under the same conditions the rate of tributyrin hydrolysis was stimulated only 1.73 × by the same concentration of Ca^{++}. Calcium stimulation of the hydrolysis rate was, however, dependent on the experimental conditions, being reduced from 1.73 × when using unemulsified tributyrin to 1.17 × when the tributyrin was emulsified in 10 % gum arabic. The stimulatory effect of Ca^{++} was also considerably reduced when strong (0.27 M), bicarbonate buffer solution was used in the manometric method, although the Ca^{++} remains in solution. Thus the rate of triolein hydrolysis was stimulated 10.5 times by Ca^{++} (0.01 M) in 0.015 M sodium bicarbonate solution, but was unaffected in 0.27 M sodium bicarbonate.

Solutions of Sr^{++} and Mg^{++} (0.01 M) stimulated the rate of hydrolysis of tributyrin less effectively than Ca^{++}, whilst Ba^{++}, Co^{++} or Mn^{++} (0.01 M) had no effect or slightly reduced the rate of hydrolysis.

On the other hand solutions of Zn^{++} and especially Cu^{++} and Hg^{++} were highly toxic to lipase even in very dilute solutions. The effect of the metal, however, varied considerably with the experimental conditions and especially with the nature of the buffer employed. Using a continuous titration method in un-buffered solution, Cu^{++} ($2 \cdot 10^{-5}$ *M*) caused 90 % inhibition of the enzyme if unemulsified tributyrin was used as substrate, but only 27 % inhibition if the tributyrin was emulsified in gelatin. This reduction of the inhibitory effect is presumably caused by a competition of gelatin with the enzyme for the Cu^{++}. When the method of FIORE AND NORD[12] was used, the effect of Cu^{++} was considerably reduced, a $2 \cdot 10^{-4}$ *M* solution having no effect on the enzyme. This method utilises a buffer containing citrate, which is an effective chelating agent for Cu^{++}.

The effect of Cu^{++} on lipase was also reduced in the presence of bicarbonate buffer, although no precipitation of the metal occurred under the conditions used. When the experiment was carried out in 0.015 *M* $NaHCO_3$, Cu^{++} ($2 \cdot 10^{-4}$ *M*) caused 88 % inhibition of lipase, but there was only 52 % inhibition in 0.28 *M* $NaHCO_3$. In the stronger solution of bicarbonate lipase could be completely protected against inhibition by Cu^{++} ($2 \cdot 10^{-4}$ *M*) by the addition of Ca^{++} (10^{-2} *M*), less effectively by equivalent concentrations of Sr^{++} or Mg^{++}, but not at all by Ba^{++} or by any other metal tested. The enzyme could also be partially protected by Ca^{++} against inhibition by Hg^{++}. After short periods of contact, *e.g.* 10 min, Cu^{++} inhibition of lipase could be partially reversed by the addition of Ca^{++}. These protection and reversal effects could only be observed in $NaHCO_3$ buffers; under other conditions no protection or reversal occurred.

Glutathione or histidine ($4 \cdot 10^{-3}$ *M*) completely protected lipase against inhibition by Cu^{++} ($2 \cdot 10^{-4}$ *M*) and also reversed the inhibition completely after the Cu^{++} had been in contact with the enzyme for periods up to 45 min. Aspartic acid and glycine were also effective protective agents of lipase against Cu^{++} inhibition, but did not restore the activity of Cu^{++} inactivated lipase. Lipase was completely protected against inhibition by Hg^{++} ($5 \cdot 10^{-5}$ *M*) by the addition of glutathione ($4 \cdot 10^{-3}$ *M*), which also reversed the inhibition of Hg^{++} after a 5 min contact period. Histidine ($4 \cdot 10^{-3}$ *M*) was, however, less effective in this case, reducing the inhibition by Hg^{++} ($5 \cdot 10^{-5}$ *M*) from 95 % to 45 %.

Histidine and aspartic acid have been reported to activate lipase[15], but these findings could not be repeated by using the purified lipase, neither amino acid having any effect on the untreated enzyme.

Metal ions such as Cu^{++} and Hg^{++} may exert their inhibitory action on enzymes by the combination with –SH groups.

In order to examine this possibility for the case of lipase, the enzyme was treated with *p*-chloromercuribenzoate (10^{-3} *M*), which combines specifically with -SH groups. This treatment caused only about 50 % inhibition of lipase. The further addition of Cu^{++} or Hg^{++} in low concentrations (10^{-4} *M*), however, still produced 100 % inhibition of the lipase. Sites other than –SH groups must therefore be involved in the combination of lipase with these metals.

The effect of –SH reagents on lipase

WEINSTEIN AND WYNNE[5] found that pancreatic lipase was weakly (22 %) inhibited by a solution of bromacetic acid (10^{-2} *M*) and 32 % by iodoacetic acid. SINGER AND BARRON[6] found that iodoacetamide ($5 \cdot 10^{-3}$ *M*) had no effect on lipase but that *p*-chloromercuribenzoate (10^{-3} *M*) caused 38% inhibition and *p*-aminophenyldichlorarsine (10^{-3} *M*) caused 62% inhibition of the enzyme. They concluded that pancreatic lipase was a typical –SH enzyme. All these experiments were carried out using relatively crude lipase preparations.

A more extensive study has now been made of the effect of a variety of typical –SH reagents on purified lipase and, although the enzyme was found to be inhibited by certain members of this group of –SH enzyme inhibitors, it was quite resistant to others (Table IV).

TABLE IV

THE EFFECT OF SULPHYDRYL REAGENTS ON LIPASE

Reagent	*Concentration (M)*	*Inhibition (%)*
Iodine	10^{-5}	100
Ferricyanide	$2 \cdot 10^{-3}$	0
Alloxan	10^{-3}	0
Iodosobenzoic acid	10^{-3}	0
Iodoacetate	$2 \cdot 10^{-3}$	0
Neoarsphenamine	10^{-3}	28
p-chloromercuribenzoate	10^{-3}	69

Most oxidising agents which convert –SH to –S–S had little or no effect, but the enzyme was very sensitive to iodine. Lipase could be protected against inhibition of iodine by glutathione, but, if iodine had had prior contact with the enzyme, no reversal was possible.

Using a purified lipase from wheat germ, SINGER[16] studied the effect of *p*-chloromercuribenzoate and *p*-aminophenylarsine oxide on the rate of hydrolysis of the triglycerides, triacetin, tripropionin and tributyrin.

The extent of inhibition of lipase by each compound was found to increase with increasing molecular weight of the substrate from triacetin to tributyrin. A similar variation of inhibition with molecular weight was observed when the mono- and the triglycerides of acetic, propionic or butyric acids were compared. In each case the inhibition of triglyceride hydrolysis was markedly greater than that of the monoglyceride hydrolysis.

The experiments described by SINGER[16] were repeated using the purified pancreatic lipase. Although the inhibition produced by *p*-chloromercuribenzoate (10^{-3} *M*) was found to be greater when using tributyrin than triacetin as substrate and to increase through the series mono-di-tri propionin as found by SINGER, the increase of inhibition with increase of molecular weight was not maintained through a series of triglycerides (Table V).

In order to explain the fact that *p*-chloromercuribenzoate inhibition of wheat lipase increases with the molecular weight of the substrate, SINGER[16] suggested that the -SH group of the wheat lipase may be located close to, but not within, the active centre. Combination of the –SH with a blocking reagent would be effective in preventing a large substrate molecule from combining with the active centre, but less effective in allowing a smaller substrate to reach the active site. Although this hypothesis may be true, an entirely different explanation of this phenomenon is,

TABLE V

INHIBITION OF LIPASE BY *p*-CHLOROMERCURIBENZOATE*
IN THE PRESENCE OF DIFFERENT SUBSTRATES

Substrate	*Inhibition (%)*
Monopropionin	19
Dipropionin	35
Tripropionin	70
Triacetin	24
Tripropionin	70
Tributyrin	61
Trioctanoin	68
Trilaurin	46
Triolein	5

* Concentration, 10^{-3} *M*.

however, possible; namely, that the different inhibitions observed with different substrates are directly proportional to the quantity of *p*-chloromercuribenzoate absorbed at the glyceride water interface.

In order to examine the possibility that partition of the *p*-chloromercuribenzoate (*p*-CMB) between the substrate and aqueous phase was an important factor in determining the extent of inhibition of the lipase, the following experiment was carried out. Equal volumes of an emulsion of tributyrin in gum arabic and *p*-CMB (0.01 *M*) were vigorously shaken and then the tributyrin centrifuged down. The supernatant was discarded and the tributyrin was shaken with an equal volume of sodium bicarbonate solution (0.05 *M*) and recentrifuged. This procedure was repeated twice. Finally the tributyrin was suspended in water to give the original volume of the emulsion. A control emulsion was shaken with water instead of with *p*-CMB and subsequently treated in a similar manner.

When the tributyrin treated with *p*-CMB was used as substrate for lipase, it caused powerful inhibition of the enzyme, despite the washing with bicarbonate solution. The inhibition observed was only slightly less than that produced by the addition of an equivalent concentration of *p*-CMB directly to the lipase, thus proving the strong affinity of the glyceride for the *p*-CMB and the importance of partition into the fat as a factor determining the inhibition.

DISCUSSION

Lipase has been found to be much less stable after treatment with Versene and other metal complexing agents and therefore the enzyme may be assumed to possess a bound metal which helps to confer stability. The nature of the metal has not been definitely established, but several lines of indirect evidence indicate that it is likely to be calcium.

This evidence may be summarised as follows: (a) the metal displaced from lipase by Versene may be most effectively replaced by Ca^{++}, (b) the curve of pH/Versene inhibition of lipase is very similar to the curve of pH/Versene bound Ca^{++} (Fig. 2), and (c) the stability of lipase exposed to a series of chelating substances decreases with increase of the chelating power of the substance for Ca^{++} through the series (Table III). Lipase does not, however, exert a specific affinity for Ca^{++} and this metal may be replaced less effectively by other metals such as Mg^{++} or Sr^{++}. This stabilising function of Ca^{++}, which does not appear to have been previously observed, is quite distinct from its activating effect on triglyceride hydrolysis.

There are, however, no reasons for believing that the originally proposed function of Ca^{++}, i.e. the removal of insoluble Ca^{++} salts of fatty acids, is not important when certain triglycerides which produce long chain fatty acids on hydrolysis are attacked by lipase. Such fatty acids are nearly insoluble in the aqueous phase and would tend to partition in the fat phase, thus slowing the rate of reaction. When short chain fatty acid glycerides are used, *e.g.* of butyric acid, Ca^{++} activation is much less than it is when long chain fatty acids are involved, but it is, nevertheless, still significant. In this case, the mechanism of Ca^{++} activation proposed above is inadequate, firstly, since the acid produced is freely water soluble and would diffuse into the aqueous phase, and secondly, since the Ca^{++} salts are soluble and ionised. A possible explanation of this phenomenon is that Ca^{++} has a special enzyme activating effect which it exerts by concentrating at the fat/water interface.

Calcium ions may therefore carry out three distinct roles in lipase action: (a) a removal of fatty acids as insoluble Ca^{++} salts in certain cases, (b) a direct enzyme activation resulting from concentration at the fat/water interface, and (c) a stabilising effect on the enzyme. The site of binding of the Ca^{++} in the stabilised enzyme is likely to be at the oxygen of the ionised carboxyl groups, since it is for these groups that Ca^{++} has the greatest affinity, having a much weaker affinity for nitrogen and sulphur.

Experiments using Cu^{++} demonstrate the impracticability of comparing results not obtained under identical experimental conditions.

In all experiments using this metal it appeared that a competition was set up between the chelating power of the buffer salt and vital enzyme centres for the Cu^{++}. The ability to remove Cu^{++} as a complex appeared to be especially powerful in the case of citrate and bicarbonate buffer and the inhibition was found to be correspondingly small.

Cu^{++} and Hg^{++} may be in part bound at –SH groups but when these groups are reacted with, and blocked by, *p*-chloromercuribenzoate, lipase is still powerfully inhibited by dilute solutions of these metals. The binding to the protein which causes the inhibition must be at other sites and the very effective reversal of inhibition observed when histidine is added suggests that a histidine residue might be an important site of metal binding.

It is possible that the activation effects observed with histidine and some other amino acids[15] may be explained on the basis of a removal of inhibitory metals such as Cu^{++}.

It has been previously suggested that lipase possesses –SH groups[5,6] and it has been proposed that they are essential constituents of the active centre[7]. The present experiments, however, have shown that the relatively pure lipase used is completely resistant to many –SH reagents and that inhibition by arsenicals and *p*-CMB is much weaker than would be expected if the –SH were actually part of the active centre. Most oxidising agents were completely without effect on the enzyme, but the strongly inhibitory effect of iodine appears to be anomalous. However, the fact that the iodine inhibition was not reversed by the addition of glutathione makes it appear probable that oxidation of –SH groups to –S–S– is not the mechanism involved in iodine inhibition of lipase, since glutathione normally reduces –S–S– to –SH and should, therefore, have reactivated the enzyme. A more likely explanation of the powerful inhibition observed with iodine is that it is a result of iodination of tyrosine residues. A possible explanation of all these experiments is that lipase definitely possesses –SH groups, but that these groups are situated adjacent to, but not in, the active site. Combination with large molecules such as arsenicals or *p*-CMB may then inhibit sterically, whilst smaller molecules such as iodoacetate are without effect. It is also possible, however, that partition of the inhibitor between the glyceride and aqueous phase may be a more important factor determining the extent of the inhibition, as has been previously discussed for *p*-chloromercuribenzoate.

For two reasons it is considered unlikely that the suggestion of Singer[16], that the effect of increased inhibition by –SH reagents observed with increased molecular weight of the substrate may be explained by a steric phenomenon, is true. Firstly, since the inhibition observed with *p*-chloromercuribenzoate only increases up to a certain molecular weight of substrate and then falls again (Table V) and secondly, the proof that the *p*-chloromercuribenzoate can partition in the glyceride to an extent sufficient to cause strong inhibition of the enzyme. The variation of lipase inhibition observed when using different substrates would then better be explained on the basis that this variation is really a measure of the different partitions of the *p*-chloromercuribenzoate between water and the insoluble substrate. This explanation is rendered the more likely in view of the recent clear demonstration of the action of lipase at the oil/water interface[17].

ACKNOWLEDGEMENTS

The author wishes to express his thanks to Dr. H. B. Pierce and his colleagues of the Biochemistry Department of the Medical School of the University of Vermont, U.S.A. for kindly providing the facilities for most of this work to be undertaken and for many helpful discussions, and to Professor A. Wormall for advice, and useful comments on this work.

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β-HYDROXY-β-METHYLGLUTARYL COENZYME A REDUCTASE, CLEAVAGE AND CONDENSING ENZYMES IN RELATION TO CHOLESTEROL FORMATION IN RAT LIVER*

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(Received February 2nd, 1960)

SUMMARY

Investigation of the intracellular localization of the principal enzymes involved in the formation and breakdown of β-hydroxy-β-methyl-glutaryl CoA (HMG CoA) in rat liver has shown that HMG CoA condensing and cleavage enzymes are both preponderantly in the mitochondria. HMG CoA reductase, which leads to the pathway of cholesterol synthesis, is in the microsomes, and is only one twentieth as active as the cleavage enzyme which leads to acetoacetate production. In spite of this unfavorable ratio, cholesterol synthesis does occur—possibly because a small amount of condensing enzyme and most or all of the reductase are in the microsomes which are low in cleavage activity.

Stimulation of cholesterol synthesis by injection of Triton, or inhibition by fasting does not importantly alter the amount or distribution of the condensing and cleavage enzymes. However, a severe depression of reductase activity in fasting suggests that this may be a significant factor in the rate limitation of cholesterol synthesis in this condition.

INTRODUCTION

When cell-free preparations of rat liver were studied under conditions in which cholesterol formation from acetate was experimentally either stimulated or suppressed, the results pointed to the existence of a major rate-controlling step at an early stage in the biosynthetic sequence. The evidence implied that the sensitive step lay in the reaction chain somewhere between acetyl CoA and mevalonic acid, and that it was

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Abbreviations used: CoA, coenzyme A; DPN and TPN, di- and triphosphopyridine nucleotides respectively; DPNH and TPNH, their reduced forms; glucose-6-P, glucose-6-phosphate; HMG CoA, β-hydroxy-β-methyl glutaryl coenzyme A; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

[*] This is publication No. 996 of the Cancer Commission of Harvard University. A preliminary report of this work was presented at meetings of the Federation of American Societies for Experimental Biology (*Federation Proc.*, 18 (1959) 20).

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mediated by an enzyme which was bound to the microsomes and dependent upon reduced pyridine nucleotide (probably TPNH)[1-4].

The pathways for the formation of cholesterol and acetoacetic acid from acetyl CoA are outlined in Fig. 1: they are identical up to the level of HMG CoA, beyond which they diverge. In the studies mentioned in the paragraph above, one method employed to suppress the conversion of acetate to cholesterol was to deprive animals of food for 24 h. Since acetoacetate formation is increased in livers of fasting animals[5,6], it can be inferred that the production of its immediate precursor, HMG CoA (see ref. 7), is not impaired in this condition. HMG CoA is also a precursor of mevalonic acid which is on the pathway leading to cholesterol. Thus the implication is that the reduction in cholesterol formation exhibited by livers of fasting animals derives not from lack of production of HMG CoA, but rather from interference with its further progress towards mevalonic acid (Fig. 1). The present investigation provides evidence in support of a lowering of HMG CoA reductase activity in such animals.

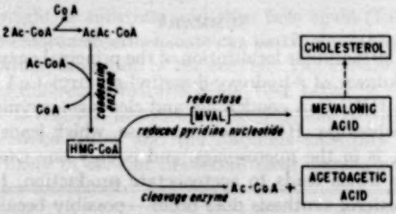

Fig. 1. Pathways for the formation of mevalonic acid and acetoacetic acid from acetyl-CoA. The following abbreviations are used: Ac–CoA, acetyl–CoA; AcAc–CoA, acetoacetyl–CoA; HMG CoA, β-hydroxy-β-methylglutaryl-CoA; MVAL, mevaldic acid.

EXPERIMENTAL

Materials and methods

Materials: We are indebted to Dr. J. Knappe for biosynthetic preparation of [1,3,5-^{14}C]HMG CoA as well as for mevalonic dehydrogenase and mevalonic kinase. [1-^{14}C]acetate was obtained from Kernreaktor Bau- und Betriebsgesellschaft m.B.H, Karlsruhe, and DPN, TPN, glucose-6-P and glucose-6-P dehydrogenase all from C. F. Boehringer, Söhne, Mannheim-Waldhof.

Animals: Young adult male Sprague-Dawley rats were allowed stock diet and water *ad lib.* Animals treated with Triton WR1339 received 1 ml of 10 % aqueous solution per 100 g of body weight intravenously 24 h before removal of the liver. Fasting animals comprised those deprived of food for 24 h.

Preparation of liver: Rats were stunned by a blow on the head and decapitated. Livers were excised, chilled thoroughly in crushed ice, rinsed in medium and put through an ice-cold tissue press. The liver pulp was homogenized as previously described[8]. For determination of HMG CoA condensing and cleavage enzymes, the medium consisted of 0.25 *M* sucrose. For other determinations it was composed of 0.1 *M* potassium phosphate buffer pH 7.4, 0.004 *M* magnesium chloride, and 0.03 *M* nicotinamide.

The supernatant fluid obtained from centrifugation of whole homogenates for 10 min at 5,000 × g was employed for routine incubations. It consisted principally of microsomes and soluble cell components, and will be designated S-5000. In more detailed cell fractionation studies a crude "nuclear" fraction (containing in addition to nuclei, red blood cells, unbroken liver cells and cell debris) was sedimented by centrifugation for 10 min at 600–700 × g and a mitochondrial fraction at 9000 × g. These procedures were carried out in a cold room at 4° in a Servall centrifuge, type SS1. A microsomal fraction was obtained after 1 h at 105,000 × g in a Spinco preparative ultracentrifuge. Each fraction was washed once by resuspension and recentrifugation. The supernatant fluid from the final centrifugation was considered to comprise the soluble cell components.

Cholesterol biosynthesis: S-5000 preparations were incubated for 2 h aerobically with [1-^{14}C]acetate, DPN, TPN and glucose-6-P. After alkaline hydrolysis cholesterol was extracted with petroleum ether, precipitated with digitonin, and its radioactivity determined in an end window counter with an efficiency of approx. 8 % (see ref. 2).

Acetoacetate formation: S-5000 preparations were incubated with labeled acetate as above. After addition of acetoacetate as carrier they were deproteinized with trichloroacetic acid and strongly acidified with HCl. The [^{14}C]acetone was collected by steam distillation in a solution of 2,4-dinitrophenylhydrazine, and the resulting hydrazone extracted with carbon tetrachloride[9]. This extract was washed thoroughly with solutions of sodium acetate and sodium bicarbonate and water, and concentrated by evaporation *in vacuo*. An aliqout was plated for determination of its radioactivity. Enzyme-free control preparations, serving as blanks, showed that no [1-^{14}C]acetate contaminated the final product.

HMG CoA Condensing and cleavage enzymes: Livers were homogenized and fractioned in 0.25 *M* sucrose. The fractions were then rehomogenized for 1 min in 0.05 *M* potassium phosphate buffer at pH 7.5 in a high speed (approx. 35,000 rev./min) blendor-type homogenizer (Edmund Bühler, Co., Tübingen), while cooled in a saline bath at —6 to —10°. In the case of the microsomes the potassium salt of EDTA was added in a final concentration of 10^{-3} *M* to promote further lysis of the particles. These procedures were found necessary for activation of particle-bound portions of the enzymes (see below). The assays were carried out by the methods previously described[7]. Preparations were incubated with Tris buffer, sodium sulfide, coenzyme A, reduced glutathione, lithium acetyl phosphate and *Cl. kluyveri* extract, the latter to provide for generation of acetyl CoA and acetoacetyl CoA. For assay of condensing enzyme an excess of cleavage enzyme from beef liver was added, and for assay of cleavage enzyme an excess of condensing enzyme from baker's yeast. The beef liver extract was free from condensing enzyme activity and the yeast extract was free from cleavage enzyme activity. At the end of a 30-min incubation the amount of acetoacetate formed was determined colorimetrically[10]. Each liver fraction was run at a minimum of two concentrations; results were considered acceptable if they fell within ± 10 % of the mean. (At extremely low levels of enzyme activity the error was somewhat larger.) A unit of enzyme activity was considered to be the amount yielding 1 µmole of acetoacetate under the conditions of this assay. Specific activity was defined as units/mg of protein.

HMG CoA Reductase: Measurement of HMG CoA reductase was carried out only on washed microsomes (see below). These were incubated for 1 h with K_2HPO_4, reduced glutathione, potassium salt of EDTA, glucose-6-P, glucose-6-P dehydrogenase, TPN, DPN, serum albumin, mevalonic dehydrogenase, and [1,3,5-^{14}C]HMG CoA. The pH was adjusted to approx. 7 with 2 *N* KOH. After incubation the reaction was stopped by heating for 2.5 min in a boiling water bath. Carrier mevalonic acid was added, and an excess of Dowex 50 (H form), and the mixture was then extracted overnight in a continuous extractor with peroxide-free ether. After addition of an excess of KOH solution the ether was distilled off *in vacuo*. The residue, containing any [^{14}C]mevalonic acid that had formed, was neutralized and reincubated with magnesium salt of EDTA, potassium bicarbonate, potassium fluoride, serum albumin, ATP and mevalonic kinase. After 2 h incubation, Dowex 50 (H form) was added, and the mixture again extracted continuously with ether overnight. The phosphomevalonic acid formed from [^{14}C]mevalonic acid remained in the aqueous phase, while residual [^{14}C]HMG was removed by the ether. Determination of the radioactivity in the aqueous phase therefore gave a measure of the amount of HMG CoA that was reduced[11]. Microsome-free blanks were run with each experiment.

Mevaldic acid disappearance: Mevaldic acid disappearance was measured as an index of mevalonic acid dehydrogenase activity. S-5000 preparations were incubated for 30 min with potassium phosphate buffer at pH 7.0, potassium fluoride, glucose-6-P, glucose-6-P dehydrogenase, TPN and mevaldic acid. The mixture was deproteinized with trichloroacetic acid, and the residual mevaldic acid determined colorimetrically by reaction with 2,4-dinitrophenylhydrazine[12].

Protein: Protein was determined by the biuret reaction[13].

RESULTS

HMG CoA condensing and cleavage enzymes: Initial experiments were carried out to determine the intracellular distribution of HMG CoA condensing and cleavage enzymes in normal liver. At first the liver fractions in 0.25 *M* sucrose were tested directly, but the resulting values were too low, since an approximately 10-fold increase in activity of both enzymes occurred in particulate prepatations that were stored at —15° (Table IA). However, the activity of particle-free supernatant fluid obtained from centrifugation at 105,000 × *g* did not increase during storage under similar conditions.

In order to activate the bound enzymes, various methods were investigated for breaking up the particles. Preparation of an acetone powder followed by extraction with phosphate buffer yielded lower values than homogenization alone. Repeated freezing and thawing resulted only in doubling the activity of condensing enzyme, compared to the 10-fold increase obtained in the same preparation on storage at —15°. High frequency oscillation with ballotini produced only two-thirds as much activation as the final method. Addition of surface-active agents was also unsuccessful: Triton WR-1339 in a final concentration of 1 % had no effect, and cholate at 0.1–1 % final concentration was strongly inhibitory. The most convenient and reproducible technique, and the one ultimately adopted for the standard assay procedure, was homogenization for 1 min in the Bühler apparatus, as described above. Although not ideal, the results indicated that a high degree of activation was obtainable under these conditions without unduly rapid destruction of the rather unstable condensing enzyme (Table IB). Confidence in this technique is engendered by the data in Tables II and III, which show that the sum of the activities of the various cellular fractions, in units/g of liver, were in reasonable accord with the level in the unfractionated homogenate.

TABLE I

ACTIVATION OF HMG CoA CONDENSING AND CLEAVAGE ENZYMES

Data are expressed as enzyme specific activities. For determination of condensing enzyme 20 μmoles of lithium acetyl phosphate, 70 μmoles of Tris buffer pH 7.9, 7 μmoles of Na_2S, 0.3 μmole of CoASH, 3 μmoles of reduced glutathione, 4 μmoles of $MgCl_2$, 7 μmoles of dipotassium-magnesium salt of EDTA, 0.01 ml of *Cl. kluyveri* extract, plus approximately 1 unit of purified HMG CoA cleavage enzyme from beef liver (specific activity 12 units/mg protein) were incubated for 30 min at 37° with aliquots of liver preparations in a final volume of 0.7 ml. For cleavage enzyme the conditions were the same, except that approximately 1 unit of condensing enzyme from a toluene autolysate of baker's yeast (specific activity 0.6 units/mg), or enriched preparations thereoff, was substituted for the beef liver preparation.

	1000 × g supernatant from homogenate in 0.25 M sucrose	
	Fresh	*5 days at —15°*
Condensing enzyme	0.006	0.078
Cleavage enzyme	0.063	0.53

	Bühler homogenate (uncentrifuged) in 0.05 M phosphate buffer			
	1 min	*2 min*	*3 min*	*4 min*
Condensing enzyme	0.067	0.061	0.051	0.055
Cleavage enzyme	0.94	0.97	0.89	0.95

It is evident from the values obtained in whole homogenates of normal livers that there was considerable variation among animals with respect to both cleavage and condensing enzymes (Tables II and III). However, the cleavage enzyme was of the order of 10 times more active than the condensing enzyme.

The intracellular distribution of the two enzymes was very similar: nearly one-third of the activity appeared in the crude "nuclear" fraction, two-thirds in the mitochondrial and a small amount in the soluble fraction. The levels in the microsomes, especially in the case of the more labile condensing enzyme, were too low to be determined reliably by this method of assay.

When animals were subjected to fasting, which strongly suppresses cholesterol formation, or were injected with Triton WR-1339, which greatly enhances it, the levels of condensing and cleavage enzymes in the small series of animals studied did not appear to rise importantly above the normal range. In any case it is clear that the slight changes that did occur, whether real or attributable to statistical variation, were in no way parallel to, or commensurate with, the enormous differences in conversion of acetate to cholesterol exhibited by S-5000 preparations from similar animals (Table IV).

The distribution of condensing and cleavage enzymes among the cellular fractions in the treated animals also remained closely similar to the normal pattern (Tables II and III).

HMG CoA reductase: Relative to cleavage enzyme, the activity of cholesterol-forming enzymes was very low. For example, when S-5000 from normal liver was incubated with [1-^{14}C]acetate under conditions favorable for cholesterol synthesis,

TABLE II

DISTRIBUTION OF HMG CoA CONDENSING ENZYME IN CELLULAR FRACTIONS

Enzyme activities are expressed as units/g of fresh liver. Specific activities are expressed as units/mg of protein. Values in parentheses are percentages of activity attributable to each fraction if the sum of the activities is taken as 100%. Procedures as in Table I.

	Homogenate	*Crude "nuclei"*	*Mitochondria*	*Microsomes*	*Supernatant*
		Normal			
	9.4				
	11.0				
	26.7				
	24.2				
		4.6	9.6		
		5.7	13.1		
		3.6	10.4		
	9.7	4.4	8.6	—	0.4
	12.3	3.6	7.9	—	0.4
	11.7	2.8	8.4	—	0.4
Average units/g	15.0	4.1 (*29*%)	9.7 (*68*%)	—	0.4 (*3*%)
Average specific activity	0.073	0.068	0.182	< 0.003	0.007
		Triton-treated			
	21.8				
		7.3	16.1		
	37.1	7.4	23.4	0.1	5.8
Average units/g	29.5	7.4 (*22*%)	19.8 (*60*%)	0.1 (*0.3*%)	5.8 (*18*%)
Average specific activity	0.138	0.125	0.325	0.006	0.08
		Fasting			
	18.9				
		6.9	14.5		
		9.1	23.5		
	26.3	11.9	29.1	0.08	4.5
Average units/g	22.6	9.3 (*26*%)	22.4 (*62*%)	0.08 (*0.2*%)	4.5 (*12*%)
Average specific activity	0.086	0.149	0.382	0.005	0.059

1.80 μmoles of acetate were converted to acetoacetate while only 0.086 μmoles were converted to cholesterol, a ratio favoring the cleavage pathway of 20 to 1. Because of this overwhelming tendency towards cleavage, it was not feasible to measure the HMG CoA reductase activity in S-5000 preparations, with the limited supply of labeled HMG CoA available. However, it had already been shown that the rate-controlling step in cholesterol formation is dependent upon the microsomes[2] which, as indicated above, contain very little cleavage enzyme. Accordingly, determinations of HMG CoA reductase were carried out only on washed microsomes. In an attempt to amplify the resulting differences, liver microsomes from animals stimulated by injection of Triton were compared with those from animals depressed by fasting.

TABLE III

DISTRIBUTION OF HMG CoA CLEAVAGE ENZYME IN CELLULAR FRACTIONS

See above Table II.

	Homogenate	*Crude "nuclei"*	*Mitochondria*	*Microsomes*	*Supernatant*
		Normal			
	123				
	185				
	175				
	105				
		33	100		
		30	85		
		26	83		
	107	41	52	0.25	2.5
	102	36	52	0.80	4.0
	139	33	102	0.70	2.3
Average units/g	134	33 (*28* %)	79 (*68* %)	0.58 (*0.5* %)	2.9 (*3* %)
Average specific activity	0.66	0.54	1.48	0.04	0.05
		Triton-treated			
	110				
		56	110		
	195	48	117	1.2	8.0
Average units/g	153	52 (*3*[illegible] %)	114 (*65* %)	1.2 (*0.7* %)	8.0 (*5* %)
Average specific activity	0.71	0.89	1.87	0.06	0.11
		Fasting			
	147				
		37	64		
		56	133		
	300	64	175	2.0	15.0
Average units/g	224	52 (*27* %)	124 (*64* %)	2.0 (*1* %)	15.0 (*8* %)
Average specific activity	0.88	0.84	2.06	0.13	0.19

TABLE IV

CONVERSION OF ACETATE TO CHOLESTEROL BY LIVER PREPARATIONS

2.0 ml of S-5000 preparations were incubated for 2 h at 37° with 20 μmoles of glucose-6-P, 2 μmoles each of TPN, and DPN and 5 μmoles of [1-^{14}C]acetate (2.5 μC) in a final volume of 2.45 ml.

Normal		*Fasting*		*Triton treated*	
Cholesterol counts/min	*(ac → ch) µmoles*	*Cholesterol counts/min*	*(ac → ch) µmoles*	*Cholesterol counts/min*	*(ac → ch) µmoles*
5,390	0.075	0*	—	18,700	0.260
6,400	0.089	5	—	19,250*	0.268
6,200	0.086	16**	—	19,600**	0.272
8,640	0.119				

* S-5000 prepared from same livers as microsomes in Expt. No. 1, Table V.

** S-5000 prepared from same liver as microsomes in Expt. No. 2, Table V.

As shown in Table V, microsomes from the Triton-treated animals were highly active in reducing HMG CoA to mevalonic acid; S-5000 preparations from the same livers were similarly active in the total synthesis of cholesterol from acetate (Table IV, footnote). In the first experiment in Table V doubling the concentration of microsomes did not increase the total conversion of HMG CoA to mevalonate. Hence even at the lower microsomal concentration the amount of HMG CoA added may not have been quite sufficient to demonstrate maximal activity. In the second experiment, however, the HMG CoA could not have been limiting since doubling its concentration did not increase mevalonate formation. The capacity of microsomes to reduce HMG CoA thus appeared to be of the order of $3-4 \cdot 10^{-3}$ μmoles/mg of microsomal protein/h in livers from Triton-treated animals.

TABLE V

REDUCTION OF HMG CoA BY MICROSOMES FROM LIVERS OF FASTING AND TRITON-TREATED RATS

For determination of HMG CoA reductase activity, washed microsomes were incubated for 1 h at 37° with 20 μmoles of K_2HPO_4, 5 μmoles each of reduced glutathione and potassium salt of EDTA, 0.3 μmole each of DPN and TPN, 8 μmoles of glucose-6-P, 0.3 mg of serum albumin, glucose-6-P dehydrogenase, mevalonic acid dehydrogenase, and [^{14}C]HMG CoA (0.01 μmole containing approximately 6000 counts/min). After adjustment of the pH to 7.0–7.2, with 2 *N* KOH the final volume was approx. 0.7 ml.

Expt. No.	*Rats*	*Microsome protein present (mg)*	*Labelled HMG CoA added (μmoles)*	*(HMG CoA → MVA) by microsomes*	
				Total activity (counts/min)	*μmoles/mg/protein/h (μmoles × 10^{-3})*
1	Triton	1.5	0.01	3480	3.9
		3.0	0.01	3430	1.9
	Fasting	1.8	0.01	0	0
		3.6	0.01	0	0
2	Triton	1.5	0.01	2850	3.2
		1.5	0.02	2860	3.2
	Fasting	1.1	0.01	40	0.06
		1.1	0.02	27	0.04

The preparations from fasting animals exhibited grossly impaired capacities to synthesize cholesterol from acetate and to reduce HMG CoA (Tables IV and V). The failure to demonstrate reductase activity could indicate either actual inhibition or loss of the enzyme itself, or an increase in HMG CoA cleavage enzyme sufficient to mask the reductase by elimination of its substrate. However, we have already shown that the activity and distribution of cleavage enzyme in terms of total capacity (*i.e.* after disruption of the particles in the Bühler homogenizer) do not differ significantly in fasting, normal and Triton-treated preparations. Further, we examined undisrupted mitochondria from similar animals to find whether fasting *per se* resulted in activation of bound cleavage enzyme. The results (Table VI) were somewhat equivocal in that the lowest as well as the highest values obtained in these intact particles were in the fasting series, but 3 out of 4 fell within the normal range. Although it cannot be entirely ruled out, the data lend little support to the idea that the cleavage enzyme in fasting animals is in a particularly active state. These findings therefore suggest that there is an actual inhibition or loss of HMG CoA reductase in the fasting animal.

The reduction of HMG CoA to mevalonic acid has been found to be mediated by

TABLE VI

CONDENSING AND CLEAVAGE ENZYME ACTIVITIES IN INTACT AND DISRUPTED MITOCHONDRIA FROM LIVERS OF NORMAL, FASTING AND TRITON-TREATED RATS

Procedures as in Table I.

Expt. No.	*Normal*		*Triton*		*Fasting*	
	Intact specific activity	*Disrupted specific activity*	*Intact specific activity*	*Disrupted specific activity*	*Intact specific activity*	*Disrupted specific activity*
			Condensing enzyme			
1	0.002	0.093	0.002	0.065	0.002	0.085
2	0.001	0.034	0.001	0.045	0.002	0.049
3	0.001		0.002		0.001	
4	0.001		0.002		0.005	
			Cleavage enzyme			
1	0.007	0.82	0.004	1.02	0.005	0.96
2	0.006	0.97	0.005	1.12	0.004	1.43
3	0.049		0.029		0.041	
4	0.049		0.029		0.077	

a single enzyme in yeast, and mevaldic acid is not an intermediate in this reaction[11, 14]. Although the same condition may obtain in mammalian tissues, the possibility of a two-step reaction involving mevaldic acid has not yet been ruled out (Fig. 1). Accordingly we measured the disappearance of mevaldic acid as an index of mevalonic dehydrogenase activity. Comparison of normal, Triton-treated and fasting animals with respect to conversion of acetate to cholesterol by S-5000 preparations yielded 8,640 counts/min in the normal, 18,700 counts/min in the Triton-treated, and 5 counts/min in the fasting respectively. Incubation of 0.5-ml aliquots of these same preparations for 30 min with mevaldic acid resulted in relatively insignificant differences: 0.256 μmoles disappeared from the normal, 0.282 from the Triton-treated and 0.266 from the fasting.

DISCUSSION

Although there are other pathways for its formation[15–17], it is probable that a major portion of the hepatic HMG CoA is produced from acetyl CoA and acetoacetyl CoA by the action of the condensing enzyme. This enzyme appears to be preponderantly associated with the mitochondria; approximately two-thirds of the condensing activity appeared in this fraction and nearly an additional third occurred in the crude "nuclear" fraction which contained enough contaminating mitochondria to account for most if not all of the activity there. The small portion of condensing enzyme found in the soluble fraction was probably derived largely from particles disrupted during homogenization and fractionation. Negligible activity was demonstrated in microsomes. However, RUDNEY, using isotopic techniques which are far more sensitive than the biochemical methods that we employed, was able to detect this enzyme and isolate it from washed microsomes, showing that at least a small fraction of it is bound to these particles. It is important to note that these sensitive isotopic tests did not demonstrate the overwhelmingly high concentration of condensing enzyme present in the mitochondrial fraction, because the high activity of cleavage enzyme prevented accumulation of HMG CoA upon which these tests depend. In the method that we employed, interference by cleavage enzyme was not a factor. It can be calculated from RUDNEY'S data[18] that in his system 0.003 μmoles of HMG were formed from acetate/mg of microsome protein/30 min. This level is at the borderline of detectability by our method of measuring condensing enzyme. We found the mitochondria to have a specific activity of 0.182 μmoles/mg, or 60 times the activity determined by RUDNEY in the microsomes.

The pattern of distribution of cleavage enzyme in the cellular fractions was closely similar to that of the condensing enzyme, indicating that it too functions mainly in the mitochondria. Possibly acetoacetate production is primarily a mitochondrial function, carried out by the condensing and cleavage enzymes that are abundant in these particles, whereas the small fraction of the condensing enzyme that is in the microsomes produces the HMG CoA which is converted to mevalonic acid by the microsomal reductase. This latter HMG CoA would be more readily available to the reductase because of the low level of cleavage enzyme in the microsomes. Such compartmentation could explain how cholesterol synthesis from acetate can occur in a tissue with a many times greater potential capacity to split HMG CoA than to synthesize it or reduce it to mevalonic acid.

The high level of HMG CoA reductase in microsomal preparations from Triton-treated and the low level from fasting rat livers is in accord with the incorporation of acetate into cholesterol in such preparations. For the Triton-treated animals we found that 0.003–0.004 μmole of HMG CoA were reduced/mg of microsomal protein/h when measured directly. For comparison, S-5000 preparations containing approx. 80 mg/of protein, approx. 30% of which was microsomal, converted 0.26 μmole of acetate to cholesterol, which is equivalent to approx. 0.002 μmole of HMG CoA reduced/mg of microsomal protein/h. Since these two sets of values for reduction of HMG CoA are of the same order of magnitude, the assumption appears valid that most if not all of the reductase is bound to the microsomes.

It has been shown that mevaldic acid can be converted to mevalonic acid by rat liver *in vitro*[19, 20], and also that addition of unlabeled mevaldic acid to a homogenate suppresses the conversion of [^{14}C]acetate to cholesterol as effectively as unlabeled mevalonic acid[21]. Further, [^{14}C]mevaldic acid is converted to steroid by rat liver homogenates much more efficiently than acetate*. In a few experiments we measured the disappearance of mevaldic acid when incubated with S-5000 preparations and found insignificant differences between normal, fasting and Triton-treated animals. This finding suggests that if in liver, unlike yeast, the reduction of HMG CoA occurs in two stages with mevaldic acid as an intermediate, the first rather than the second reductive step is the critical one.

From the evidence presented above, the diminution in cholesterol formation from acetate that is found in livers of fasting rats appears to be associated with a decrease in HMG CoA reductase acitvity. This finding is no doubt relevant to the operation of the physiological control of hepatic cholesterol synthesis, but it should be emphasized that comparison of isclated systems with those operating in the intact animal involves the risk of minimizing variables that are readily eliminated *in vitro* and yet may be paramount *in vivo*. The partially inactive state of the condensing and cleavage enzymes in intact particles is a case in point. Its physiological significance cannot be properly evaluated on the basis of the maximum capacities which are measured after the particles have been disrupted. Alterations in fragility of particles in altered physiological states may interfere with accurate determinations in non-disrupted particles. This may partially explain the variability in our own experiments, as well as the findings of Migicovsky that mitochondria from fasting animals contain an inhibitor of cholesterol synthesis[22]. Thus the final limitation in the whole animal may be effectively brought about either by alteration of a critical step on the direct pathway, or of some ancillary step operating to alter the availability of essential substrates or cofactors. Whether the lowering of reductase activity in fasting is coincident with or consequent to alterations in these ancillary steps requires further study.

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* We are indebted to Dr. J. GRANT for this unpublished information.

ACKNOWLEDGEMENTS

We are grateful to Miss E. Ringelmann and Miss I. Kessel for general assistance in expediting this project. Particular thanks are due to Dr. U. Henning for helpful advice concerning the assay of acetoacetate, and HMG CoA condensing and cleavage enzymes. Especially we are indebted to Dr. J. Knappe for generous and painstaking expenditure of time, effort and laboriously-prepared materials, making possible the determinations of mevaldic acid disappearance and reduction of HMG CoA. This investigation was supported in part by a grant from the American Cancer Society and Grant No. C-2146(C5) of the United States Public Health Service.

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ACTIVATION OF THE HILL REACTION BY AMINES*

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(Received August 25th, 1959)

SUMMARY

1. Unidentified non-metal bases from plant and animal tissues, ammonia, and a number of monofunctional amines increase the rates at which ferricyanide and other oxidants are reduced by illuminated chloroplasts. The relatively polar polyfunctional amines are completely inactive.

2. The amount of activation of the chloroplast reactions is a function of the concentration of the unionized amine. The reciprocal of the rate increase plotted against the reciprocal of the concentration of the amine (methylamine) gives a straight line. Moreover the action of the amine is completely reversible. However there is no evidence that any of the amine is consumed.

3. The electron transport system operative in the absence of amines and the electron transport system activated by amines utilize different pathways. The amine-insensitive system is very stable below pH 7.0, is somewhat labile at pH 7.8, and does not reduce FMN at appreciable rates. The system activated by amines is very labile below pH 7.0, is fairly stable at pH 7.8, readily reduces FMN, and reduces all oxidants at rates which are several times greater than the maximum rate of the amine-insensitive system.

4. The amine-activated system is closely related to the system which is activated by the presence of ADP, orthophosphate and magnesium ions: the two systems disappear simultaneously on storage of the chloroplasts; the activations are not additive if optimal amounts of amine have been used; and the presence of amines prevents the formation of ATP.

5. The stoichiometry of the phosphorylating reaction and the manner in which amines might affect this reaction are discussed.

INTRODUCTION

Several years ago the author[1] investigated the reduction of flavins by illuminated chloroplasts. In the course of these studies it was discovered that the crude nucleotide fraction of yeast extracts stimulated not only the reduction of flavins but also the

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[*] Contribution No. 162.

Abbreviations used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide; TPN^+, triphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; IDP, inosine diphosphate.

reduction of all the classical HILL reaction oxidants. It was subsequently shown that the catalyst or activator could be adsorbed on cation exchange resins and eluted there from with dilute acids. Moreover, the activating substance was destroyed by boiling the preparation with nitric acid. Consequently, it seemed probable that an amine was involved.

At this point in the investigation we became aware that KROGMANN, JAGENDORF AND AVRON[2] had observed a somewhat similar stimulation of ferricyanide reduction by ammonium sulfate. Suspecting that the ability to stimulate chloroplast reactions might be a general property of non-metal bases, we tested the efficacy of a number of amines. Many of these caused marked increases in the rate of reduction of various oxidants. Since the activation seemed to be quite unspecific, attempts to identify the biologically produced active substance were set aside in favor of an investigation of the mechanism of activation.

In the experiments described below we have attempted to show that there are quite distinct amine-sensitive and amine-insensitive electron transport systems in chloroplasts. We have shown that the amine-sensitive pathway is closely related to the phosphorylation-coupled system described by AVRON *et al.*[3,4] and ARNON *et al.*[5]. Some evidence which may bear on the relative reducing potentials of the amine-sensitive and the amine-insensitive pathways is presented and the nature of the competition between these systems is described. Preliminary experiments measuring the efficiency of ATP formation and the effect of amines on this efficiency are also described.

METHODS

Chloroplasts from greenhouse-grown peas (*Pisum sativum*), variety Alaska, were used in all the experiments described below. Freshly picked pea leaves from young plants were placed in a chilled mortar with approximately isotonic KCl or sucrose buffers and ground for about 1 min with a pestle. The resulting mash was strained through glass-wool into chilled centrifuge tubes and the chloroplast suspension was spun for about 3 min at 3000–4000 × *g*. The chloroplasts which had been thrown down were re-suspended in ice-cold buffer, spun down again and again suspended in buffer. This final suspension was filtered through glass-wool and stored in a vessel well submerged in an ice bath. Aliquots of the suspension were made up to 80 % acetone and centrifuged, and the chlorophyll concentration was determined from the extinction coefficients of MACKINNEY[6]. A number of different buffers were used for isolation of the chloroplasts and as part of the reaction medium. These are described separately for each experiment.

In most of the experiments the reduction of ferricyanide was followed by measuring the decrease in O.D. of the reaction mixture at 420 mμ. The apparatus is shown diagrammatically in Fig. 1a. The monochromatic beam from a Beckman D.U. spectrophotometer passed through the 16 holes of a disc rotating at 1800 rev./min. The "chopped" beam then passed through the reaction vessel (a standard 1-cm cuvette) through a liquid filter, and finally impinged on a photomultiplier tube. By means of a load resistor and a capacitor the alternating component of the tube's output was fed into the microphone input of a standard audio amplifier. A small capacitor was placed across the input to the amplifier to eliminate most of the predominately high frequency photomultiplier noise. The voltage from the amplifier's output transformer (500 Ohm tap working into a 1000 Ohm load) was fed to an LC series resonant circuit tuned to 480 cyc./sec. The voltage across the inductance, which was about 5 times the input voltage and very low in noise, was rectified, smoothed and fed through a voltage divider to a Brown recorder. The actinic light consisted of an intense beam from a 300-W projector for 35 mm transparencies and was filtered through a red glass filter which transmitted only wave-lengths longer than 620 mμ. The projector was powered by ripple-free direct current. Light intensities were varied by the use of neutral filters. The liquid filter in the monochromatic beam (F) consisted of a solution made by adding copper sulfate to a concentrated solution of ammonium carbonate. This filter was practically opaque to the red actinic light, but transmitted nearly all of the blue measuring beam. Thus practically none of the actinic light scattered by the chloroplasts could reach the photomultiplier tube, and if any did, it was unmodulated and could give no response through the alternating current amplification system. The intensity of the actinic light was sufficient to saturate even at the highest reaction rates. At the beginning of each run the complete reaction mixture (2.0 ml) was placed in the cuvette and the slit-width of the monochromator was varied until the "optical density" indicated on the recorder equalled the known O.D. of the amount of ferricyanide present. Thus, regardless of the true O.D. of the suspension, the recorder registered at all times throughout the experiment only that part of the O.D. due to ferricyanide. Because of the tremendous cumulative gain of the photomultiplier tube, amplifier and tuned circuit and the low noise level of the overall system, it was possible to use very turbid suspensions and high concentrations of ferricyanide when this was necessary for special purposes. However, the experiments described here were carried out with relatively low concentrations, and hence with fairly transparent reaction mixtures.

This photometric equipment is quite versatile, because two independent methods of separating the effects of the actinic light and the measuring light are employed: separation by the use of complementary filters and separation on the basis of modulation. Thus with appropriate changes in the filters other photochemical reactions may be studied. For instance, by using a solution of methylene blue in the liquid filter (F) and a 340 mμ measuring beam, it was possible to follow directly the effect of light on the reduction of TPN^+ by chloroplasts.

All the reactions reported here were conducted with constantly rising temperatures. However, probably because of the short duration of the runs (usually 2 min), there seemed to have been no serious loss of reproducibility. The starting temperature with ice-cold reagents pipetted into a cuvette at room temperature was usually 6 to 8° and the final temperature was from 10 to 14°.

In some experiments oxygen production or consumption was measured instead of oxidant disappearance. For these experiments manometers and a special illuminated bath were used. The determinations were made over a longer time, usually 15 min, and the temperature was held at 8°. Reflector type incandescent lamps provided a saturating or nearly saturating light intensity.

The amount of ATP formed during the reduction of ferricyanide was determined through the almost stoichiometric formation of glucose-6-phosphate and TPNH. The TPNH was measured by its light absorption at 340 mμ, by assuming a molar extinction coefficient of $6.3 \cdot 10^3$. The 2.0-ml reaction mixture contained a tris(hydroxymethyl) aminomethane–phosphate buffer at pH 7.9, AMP, ADP, sodium fluoride, hexokinase, glucose, magnesium sulfate, potassium ferricyanide and freshly isolated chloroplasts containing 30–60 μg chlorophyll. After the reaction the mixture was transferred to a centrifuge tube and the cuvette was rinsed twice with 1.0 ml $M/15$ phosphate buffer, pH 7.3. The reaction mixture and washings were combined and immediately heated in a boiling water bath for 5 min. After cooling cysteine (2 μmoles in 0.2 ml), TPN^+ (1.0 μmoles in 0.1 ml) and glucose-6-phosphate dehydrogenase (0.15 mg in 0.1 ml) were added and the suspension was centrifuged until it was clear. The O.D. at 340 mμ were read after 6 h at room temperature. Since the O.D. resulting from 1.0 μmoles of TPNH in the total volume of 4.4 ml is 6.3/4.4 or 1.43, the observed O.D. were divided by 1.43 to give the number of μmoles of TPNH formed. The O.D. resulting from the addition of known amounts of ATP indicated a good recovery of TPNH. However the blanks, that is the O.D. of reaction mixtures without added ATP and without illumination, were high and somewhat erratic. For this reason the data on ATP formation must be considered only semi-quantitative until further investigations of the reaction system have been completed.

The adenosine phosphates (ATP, ADP and AMP), the triphosphopyridine nucleotide (TPN^+), and the hexokinase were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. The glucose-6-phosphate dehydrogenase was obtained from Sigma Chemical Company, St. Louis, Mo. The catalase was prepared from horse liver by the method of KEILIN AND HARTREE[7].

EXPERIMENTAL

Activation of the HILL *reaction by naturally occurring bases*

At the Annual Meeting of the American Society of Plant Physiologists in 1957, the author reported that basic substances capable of increasing HILL reaction rates manyfold occurred in aqueous extracts of spinach leaves, horse liver and baker's yeast. These substances were destroyed by boiling nitric acid. Amines, including ammonia, are often destroyed by hot nitric acid in the presence of organic matter, presumably because some of the nitric acid is reduced to nitrous acid which readily attacks amines. Consequently it was suspected that the unknown active substances were amines.

Figs. 1b, c and d summarize the data presented at the 1957 meeting. The catalytic bases were obtained in the following manner: Tissue was ground, mixed with water and boiled for a few minutes. The filtrate was extracted with phenol. Ether insoluble materials such as nucleotides and other salts were extracted from the phenol into water by the addition of several volumes of water saturated ether. The water phase was then passed alternately through several columns of Amberlite IR_4B (quaternary amine) resin and Amberlite IRC_{50} (carboxylic acid) resin. The active bases were eluted from the carboxylic acid resin with 1.0 N acetic acid and the acetic acid was removed by distillation at reduced pressure. The dry residue was taken up in a small volume of water, neutralized with dilute NaOH and tested in the spectrophotometric apparatus depicted in Fig. 1a. Very small amounts of active material were obtained by this laborious procedure.

Figs. 1b and c show that the reduction of ferricyanide by illuminated chloroplasts was about 6 times as rapid in the presence of these catalytic bases as in their absence. However, from Fig. 1c it can be seen that the ability of the chloroplasts to respond to the bases was quite labile. After 1.5 h at room temperature addition of the erstwhile activators had no effect. In the same time the uncatalysed rate remained completely unchanged.

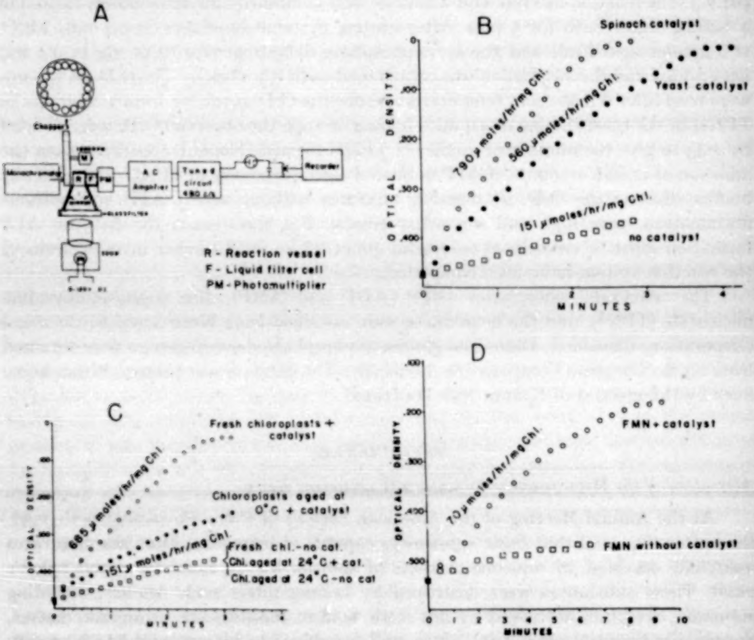

Fig. 1. a. Diagram of the reaction vessel, light sources and associated photometric equipment. The actinic light was filtered through red glass which transmitted only those wave lengths longer than 620 mμ. The complementary liquid filter (F) in the monochromatic measuring beam (420 mμ) consisted of a solution of cupric ammonium carbonate. See METHODS. b. The reduction of ferricyanide by illuminated chloroplasts and the catalytic effects of bases extracted from yeast and spinach leaves. Buffer: 0.033 *M* sodium phosphate, pH 7.0, containing 16 g KCl/l. Potassium ferricyanide 0.89 μmole. Total volume 2.0 ml. c. The effect of ageing chloroplast preparations on the ability of the chloroplasts to respond to the catalytic bases. Reaction system as in Fig. 1b. Bases extracted from spinach leaves as described in text. Chloroplasts aged 20 h at 0° or 1.5 h at 24°. d. The anaerobic reduction of flavin mononucleotide (FMN) by illuminated chloroplasts. Buffer as in Fig. 1a. Catalase in large excess and ethanol to 2 % added. See text for a description of the overall reaction.

The anaerobic reduction of flavin mononucleotide (Fig. 1d) requires special comment. In order to demonstrate the reduction of such autoxidizable substances by chloroplasts, an accessory hydrogen peroxide trapping system is required. Thus

$$\text{FMN} + \text{H}_2\text{O} \xrightarrow[\text{light}]{\text{cloroplasts}} \text{FMNH}_2 + \tfrac{1}{2}\text{O}_2 \qquad (1)$$

$$\text{FMNH}_2 + \text{O}_2 \longrightarrow \text{FMN} + \text{H}_2\text{O}_2 \qquad (2)$$

$$\text{H}_2\text{O}_2 \longrightarrow \text{H}_2\text{O} + \tfrac{1}{2}\text{O}_2 \qquad (3)$$

and consequently there is no net reaction. However, in the presence of ethanol and excess catalase reaction (3) no longer occurs[8]. Thus there is a net uptake of oxygen in aerobic systems and a net reduction of flavin in anaerobic systems. The experiment illustrated by Fig. 1d was therefore conducted in an anaerobic cuvette with added ethanol and catalase. It is clear that the activating bases were necessary for FMN reduction.

The reduction of oxidants in the presence of illuminated chloroplasts is ordinarily interpreted as evidence of the HILL reaction, that is, as evidence of the functioning of one part of the photosynthetic process. However, when we observed these strikingly enhanced rates of ferricyanide reduction, we had some misgivings. Our spectrophotometric data, which represented the reduction of very small amounts of fericyanide, could easily have indicated uninteresting, non-biological photosensitized oxidations. Consequently it seemed imperative that the conclusions drawn from these experiments should be confirmed (a) by showing that the higher rates of reduction could be sustained, and (b) by demonstrating a similar increase in the rate of oxygen production. Therefore we resorted to the use of manometers and measurements were continued over longer periods. The experiment illustrated in Table I successfully established both points; oxygen production was stimulated and the stimulation persisted for at least 15 min.

TABLE I

THE EFFECT OF A CATALYST FROM SPINACH LEAVES ON THE RATE OF OXYGEN EVOLUTION BY ILLUMINATED CHLOROPLASTS

Manometric data. Buffer: 16 g KCl/l, 0.033 *M* sodium phosphate, pH 7.0. Potassium hydroxide (10 %) in center wells. Temperature 8°. Incandescent lights. The light intensity and the chlorophyll content of the chloroplasts were not measured.

Oxidant	*Oxygen evolution (µl/15 min)*	
	No catalyst	*Catalyst added*
Ferricyanide (22 µmoles)	6.8	32
Quinone (10 µmoles)	12.7	24
FMN (5 µmoles) + Catalase + Ethanol	0	—20.2*

* The rate of net oxygen uptake in this system equals the rate of oxygen production by the chloroplasts[1].

Two other facts were established by the data of Table I. Oxygen production with benzoquinone as oxidant was increased by the catalytic bases. Moreover FMN reduction, as measured by oxygen uptake in the presence of the peroxide trapping catalase and ethanol, was not only increased by these catalysts; it depended on them completely.

Activation of the HILL *reaction by amines*

Since the naturally occurring activators of the HILL reaction had the properties of amines, a number of known amines were tested on the ferricyanide illuminated chloroplast system. Table II summarizes some of the results. In other experiments it was found that glycine, choline and aniline were inactive while tryptamine and the ethyl ester of glycine were active. Obviously tris(hydroxymethyl)aminomethane, which has long been used interchangeably with phosphate as a buffer for chloroplast reactions, is also inactive. A consistent pattern is evident in these data. All "monofunctional" amines, with the exception of the very weakly basic aniline, stimulated ferricyanide reduction while all of the "polyfunctional" amines tested were inactive. The most conspicuous difference in the properties of these two groups of amines is the degree of polarity; the monofunctional amines in their unionized forms are quite lipid soluble, while none of the polyfunctional amines could be expected to have any lipid soluble form at or near neutrality.

TABLE II

THE EFFECT OF VARIOUS AMINES ON THE RATE OF REDUCTION OF FERRICYANIDE BY ILLUMINATED CHLOROPLASTS

Ferricyanide reduction was determined spectrophotometrically. The reaction mixture (total volume 2.0 ml) consisted of buffer (16 g KCl, 0.02 moles tris(hydroxymethyl)aminomethane and 0.02 moles sodium dihydrogenphosphate/l adjusted to pH 7.5), potassium ferricyanide (0.89 µmole), and chloroplasts containing 22.5 µg chlorophyll. The amines were all 0.002 *M* Reaction time: 2.0 min.

Amine used	*Rate of ferricyanide reduction (moles × 10^{-4}/mg chl./h)*	*Increase due to amine (moles × 10^{-4}/mg chl./h)*
(A) Monofunctional amines		
None	2.9	—
Ammonia	8.1	5.2
Methylamine	5.4	2.5
Ethylamine	5.4	2.5
n-hexylamine	3.9	1.0
Cyclohexylamine	3.9	1.0
Diethylamine	3.9	1.0
Triethylamine	3.6	0.7
(B) Polyfunctional amines		
None	2.7	—
1,2-diaminoethane	2.7	0
1,5-diaminopentane	2.7	0
2-aminoethanol	2.7	0
Lysine	2.7	0
Arginine	2.7	0
2-amino-1,2,4-triazole	2.7	0
Hydroxylamine	1.8	—0.9 (inhibitory)

The status of triethylamine is crucial if one is to postulate mechanisms by which the activation occurs; tertiary amines enter into very few of the reactions characteristic of primary and secondary amines. Therefore the triethylamine was carefully purified, first by incubation with benzoylchloride to remove any reactive primary and secondary amines and then by crystallization of the hydrochloride from a mixture of benzene and alcohol. The purified triethylamine still stimulated ferricyanide reduction several-fold when large amounts were used.

Table III shows that, as in the case of the unknown naturally occurring activator, the stimulation of ferricyanide reduction by ammonia or methylamine was accompanied by the expected stimulation of oxygen production. Again oxygen production with benzoquinone as oxidant was similarly accelerated and again the reduction of FMN depended on the presence of the activator.

TABLE III

THE EFFECT OF METHYLAMINE AND AMMONIA ON THE RATE OF OXYGEN EVOLUTION BY ILLUMINATED CHLOROPLASTS

Manometric data. Buffer: 0.25 *M* sucrose, 0.01 *M* NaCl, 0.035 *M* sodium phosphate; pH 7.2. Potassium hydroxide (10%) in the center wells. Temperature 8°. Incandescent lights. The light intensity and chlorophyll content of the chloroplasts were not measured

Oxidant	*Oxygen evolution (µl/15 min)*		
	No catalyst	*Methylamine (0.03 M)*	*Ammonia (0.002 M)*
Ferricyanide (15 µmoles)	8	12	16
Quinone (6.5 µmoles)	12	27	40
FMN (1.25 µmoles) + Catalase + Ethanol	0	—10*	—13*

* The rate of net oxygen uptake in this system equals the rate of oxygen production by the chloroplasts[1].

Table IV shows that the stimulation by methylamine is a function of the concentration of the unionized amine. It shows also that the mechanism of stimulation is not otherwise dependent on the hydrogen ion concentration of the medium. This dependence on the unionized molecule is consistent with the earlier observation that the polar amines are inactive since all the ionized molcules also would be polar. When allowance is made for the different p*K*'s, most of the differences between the activities of the low molecular weight amines and ammonia disappear. However, the larger amines, such as tryptamine and triethylamine, are definitely less active even on the basis of the concentration of unionized molecules.

The reciprocal of the activation plotted against the reciprocal of the amine concentration gives a straight line (Fig. 2). This suggests that the amine somehow functions as a reactant in the system catalysed. The stimulation by methylamine is

TABLE IV

DEPENDENCE OF THE METHYLAMINE STIMULATION OF THE AMOUNT OF UNIONIZED AMINE

Ferricyanide reduction was determined spectrophotometrically. The reaction mixture consisted of buffer, potassium ferricyanide (0.89 µmole), methylamine in the amounts specified, and chloroplasts containing 27.5 µg chlorophyll. The three buffers contained 0.25 *M* sucrose, 0.01 *M* NaCl, 0.033 *M* sodium phosphate, and 0.016 *M* tris(hydroxymethyl)aminomethane. They were adjusted to pH's 6.8, 7.3 and 7.8 with HCl or NaOH. The total volume of the system was 2.0 ml and the duration of each experiment was 2 min.

Concentration of amino ($M \times 10^{-4}$)	*pH*	*Concentration of unionised amine ($M \times 10^{-4}$)*	*Increase in rate of ferricyanide reduction (µmoles/mg chl/h)*
60	6.8	8.7	530
20	7.3	9.1	424
6	7.8	8.7	500
30	6.8	4.35	320
10	7.3	4.45	275
3	7.8	4.35	344

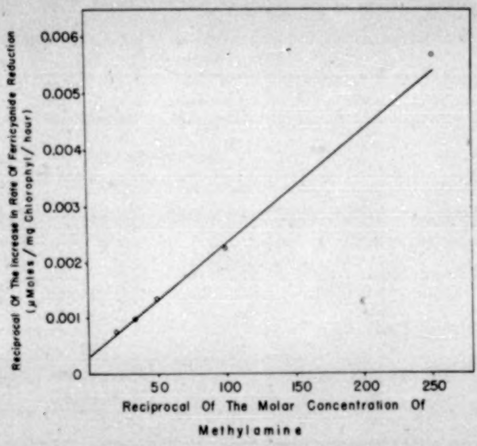

Fig. 2. The increase in rate of ferricyanide reduction by illuminated chloroplasts as a function of the concentration of methylamine. Buffer: 0.25 M sucrose, 0.01 M NaCl, 0.035 M sodium phosphate, pH 7.2. Potassium ferricyanide: 0.89 μmole. Chlorophyll content of chloroplasts: 18 μg. Total volume: 2.0 ml. Reaction time: 2.0 min. Rate without methylamine: 101 μmoles ferricyanide reduced/mg chlorophyll/h.

also completely reversible. Chloroplasts may be treated with very high concentrations of methylamine and then washed free thereof without being changed in any detectable manner; the abilities to reduce ferricyanide in the absence of amines, to be stimulated by amines and to be stimulated by the components of the phosphorylating system are retained unaltered. One is therefore tempted to suppose that the amine is directly involved in some of the reactions associated with electron transport. However, it has not been possible to obtain evidence of amine disappearance. Concentrations of ammonium sulfate which represent much less than one ammonia molecule for each molecule of ferricyanide reduced gave appreciable and persistent stimulations. Moreover after the ferricyanide had been reduced, the amount of ammonia, as measured by Nessler's reagent, had not diminished.

Amines and the phosphorylation of ADP

Avron, Krogmann and Jagendorf[3] and Arnon *et al.*[5] have shown that the rate of reduction of ferricyanide by illuminated chloroplasts is considerably increased by the presence of the components of a system which phosphorylates ADP. The stimulation requires the simultaneous presence of ADP, orthophosphate and magnesium ion[3]. They therefore concluded that part of the electron transport is obligatorily coupled to ADP phosphorylation. On the other hand, Krogmann, Jagendorf and Avron[2] have shown that very high rates of ferricyanide reduction may be achieved without concomitant phosphorylation if ammonium sulfate is present. This they have attributed to an "uncoupling" of phosphorylation by the ammonium sulfate. We have been able to confirm all these observations. We have found that the rate of ferricyanide reduction by freshly made pea chloroplasts preparations may be increased approximately threefold by the presence of ADP (or IDP), phosphate and magnesium ion. The exclusion of ADP, phosphate or magnesium prevented the increase. And, of course, ammonia and many amines activate the process of ferricyanide reduction.

There are thus at least two means by which increased electron transport capacity may be conferred on chloroplasts—the addition of amines or the addition of the components of the phosphorylating system. The series of experiments illustrated by Fig. 3 was designed to establish the similarities and dissimilarities of the two activations and to compare the activated condition with the unstimulated condition. Chloroplasts were isolated and stored at 0° in buffers of different pH. At intervals the rates of ferricyanide reduction were determined (a) in the presence of methylamine, (b) with ADP, phosphate and magnesium ion, (c) with both methylamine and the phosphorylating system and (d) without addenda. Obviously the stimulation by methylamine is akin to the stimulation by the phosphorylating system since the abilities of the chloroplasts to respond show the same pattern of decay. Both the amine-activated electron transport and the phosphorylation-dependent transport involve pathways which are labile below pH 7.0, but fairly stable at pH 7.8. Furthermore, the rate of ferricyanide reduction in the presence of both methylamine and the phosphorylating system was the same as the rate with methylamine alone. Since the rates of transport by the two activated pathways never seemed to be additive when optimal amounts of amine were used, the pathways must share a rate limiting step. Consequently they must compete for electrons and one would expect an apparent "uncoupling" of phosphorylation and ferricyanide reduction if the amine-activated transport competes on favorable terms with the phosphorylating pathway. Alternatively the amine might actually uncouple the phosphorylation step by modifying the structure or environment of some enzyme in a manner which permitted an electron transfer without the normally associated phosphorylation.

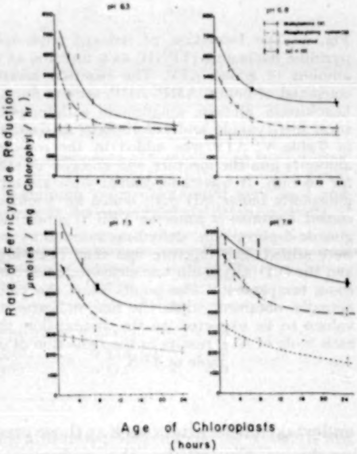

Fig. 3. The rate of ferricyanide reduction by illuminated chloroplasts as a function of the age of the chloroplast preparations and the pH of the storage medium. The chloroplasts were stored at 0° in buffers containing 0.4 *M* sucrose, 0.01 *M* NaCl, 0.01 *M* tris(hydroxymethyl)aminomethane, and 0.01 *M* sodium dihydrogen phosphate. These buffers were adjusted to pH's 6.3, 6.8, 7.3 and 7.8 by addition of HCl or NaOH. All reactions were conducted at pH 7.9 in buffer containing 0.4 *M* sucrose, 0.01 *M* NaCl, 0.05 *M* tris(hydroxymethyl)aminomethane and 0.05 *M* sodium phosphate. Potassium ferricyanide (0.89 μmole) was added to each reaction mixture and its disappearance was determined by following the decrease in O.D. at 420 mμ. Final volume: 2.0 ml. Reaction time: 2.0 min. Methylamine was added to give a final concentration 0.02 molar. The "phosphorylating system" consisted of the following addenda: ATP (2 μmoles), hexokinase (0.4 mg), glucose (40 μmoles), and magnesium sulfate (15 μmoles). The length of the vertical lines indicates the error introduced by reading the recorder charts to the nearest half millimeter.

Either mechanism of amine activation—true uncoupling or effective competition from a process parallel to the phosphorylating process—would decrease the efficiency of phosphorylation, perhaps even to the point of suppressing phosphorylation altogether. In order to determine the amount of phosphorylation in terms of the electron transport and to investigate the effect of amines thereon, experiments were undertaken in which the formation of ATP and the reduction of ferricyanide were measured simultaneously. ATP production was determined through the hexokinase-catalysed formation of glucose-6-phosphate and the subsequent reduction of an equivalent amount of TPN+ by glucose-6-phosphate dehydrogenase. Fig. 4 shows that ATP added to the complete reaction mixture may be measured practically quantitatively as TPNH. Unfortunately there was a large and rather variable amount of TPN+ reduction in the absence of added ATP and in the dark. Part of this TPN+ reduction was traced to a slight contamination of the ADP with ATP. Another part may have been due to the myokinase activity of the preparations; to inhibit this fluoride and AMP were added[9]. A more systematic investigation of these background reactions must be undertaken before data such as those presented in Tables V and VI should be considered conclusive. Nevertheless the production of ATP as a concomitant of the reduction of ferricyanide—inferred by AVRON *et al.*[3,4] and ARNON *et al.*[5] from the existence of an ADP-dependent phosphate uptake, and measured directly by KROGMANN *et al.*[2]—is again confirmed by the data of Table V. The data of Table V also support the concept of "coupled phosphorylation" by showing a dependence of ATP formation on the presence of an oxidant. As expected methylamine prevented ATP formation.

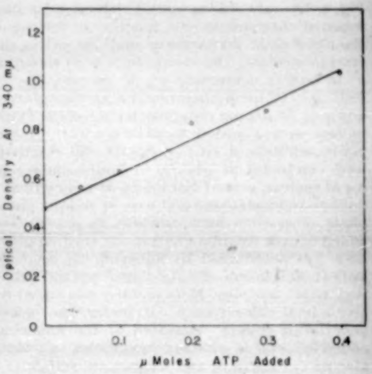

Fig. 4. The formation of reduced triphosphopyridine nucleotide (TPNH) as a function of the amount of added ATP. The reaction mixture consisted of buffer, AMP, ADP, sodium fluoride, hexokinase, glucose, magnesium sulfate, potassium ferricyanide and chloroplasts as described in Table V. ATP was added in the indicated amounts and the mixture was allowed to stand for 10 min. It was then diluted with 2.0 ml of phosphate buffer (pH 7.3), boiled for 5 min and cooled. Cysteine (2 μmoles), TPN (1 μmole) and glucose-6-phosphate dehydrogenase (0.15 mg) were added. The mixture was then centrifuged and the O.D. at 340 mμ was measured after 6 h at room temperature. The points show the values actually obtained, while the line indicates the values to be expected on the assumption that each mole of ATP results in the reduction of one mole of TPN+.

Enough data were accumulated to permit a tentative estimate of the quantitative relation of ATP formation to electron transport, and hence a reasonable prediction of the stoichiometry of the phosphorylation reaction. However, the estimate is very much complicated by the known presence of a system which operates in the absence of phosphorylation—the amine-insensitive, ADP-independent "basic" HILL reaction. Obviously an evaluation of the competition for electrons between the phosphorylating system and the "basic" system is necessary before the efficiency of the phosphorylating reaction can be determined even approximately. Such an evaluation may be approached by comparing ATP yields at different light intensities.

TABLE V

THE INFLUENCE OF FERRICYANIDE AND METHYLAMINE ON THE FORMATION OF ATP BY ILLUMINATED CHLOROPLASTS

ATP was measured through the formation of glucose-6-phosphate and the subsequent reduction of an equivalent amount of TPN by glucose-6-phosphate dehydrogenase. The complete system consisted of buffer, AMP (6 μmoles), ADP (6 μmoles), sodium fluoride (20 μmoles), hexokinase (0.3 mg), glucose (40 μmoles), magnesium sulfate (20 μmoles), potassium ferricyanide (0.89 μmole) and chloroplasts containing 34 μg chlorophyll. The total volume was 2.0 ml. 28 μmoles of methylamine were used. The buffer consisted of 0.4 *M* sucrose, 0.05 *M* tris(hydroxymethyl)aminomethane, 0.01 *M* NaCl, 0.25 *M* KH_2PO_4. The pH was adjusted to 7.9.

Experiment	*O.D. at 340 mμ*	*TPNH equivalents (μmoles)*	*ATP formed due to light (μmoles)*
Complete system (dark)	0.220	0.154	—
Complete system (light)	0.660	0.460	0.306
Complete system + Methylamine (light)	0.255	0.178	0.024
System less ferricyanide (light)	0.295	0.206	0.052

TABLE VI

THE EFFICIENCY OF ATP FORMATION

Reaction system as in Table V. In the presence of optimal amounts of methylamine the limiting light intensity gave 44 % of the rate with saturating light. The saturating light was eight times more intense than the limiting light. Chloroplasts containing 45 μg chlorophyll were used and 0.89 μmole of ferricyanide were reduced. Values represent the average of six determinations.

Experiment	*O.D. at 340 mμ*	*TPNH equivalents (μmoles)*	*ATP due to light (μmoles)*	*Moles ferricyanide reduced/ mole ATP formed*
Dark controls	0.394	0.275	—	—
Limiting light	0.842	0.592	0.315	2.8
Saturating light	0.552	0.386	0.111	8.0

We suspect that the combined phosphorylating and non-phosphorylating systems are rate-limiting at high light intensities, because amines acting at or near the phosphorylating electron transfer cause a considerable additional activation (Fig. 2). Consequently at high light intensities both processes probably operate without serious competition and near capacity. Therefore the contribution of each may be deduced from the rates with and without the presence of the components of the phosphorylating system. For instance, if at high light intensity the rate in the presence of ADP, phosphate and magnesium ion is twice the "basic" rate, it is probably safe to assume that one half of the electrons are transferred by the phosphorylation-coupled pathway, and that the efficiency of the phosphorylation-coupled pathway. may be taken as twice the overall efficiency. KROGMANN *et al.*[2] have used this method. On the other hand, the two processes of electron transfer must compete at very low light intensities, although the nature of this competition cannot be predicted *a priori*. Actually, we find that the overall efficiency of phosphorylation with severe competition (*i.e.*, low light intensity) is similar to the calculated value obtained in the manner described above for conditions of no competition (high light intensity). This can only mean that the phosphorylating pathway competes for electrons (or hydrogens) very favorably with the ADP-independent pathway. Our preliminary observations (Table VI) indicate that the transfer of somewhat less than 3 electrons was required for the formation of one ATP. This value probably approaches the value for the coupled system alone, and agrees reasonably well with the values reported by ARNON *et al.*[5], KROGMANN *et al.*[3] and AVRON *et al.*[4]. The determination by AVRON *et al.* seems also to have been made under conditions which would produce competition between the two systems. From their data a rate of 200 μmoles ferricyanide reduced/mg chlorophyll/h may be calculated; this probably represents considerably less than 50 % of the maximum attainable reaction velocity and therefore suggests the use of rather low light intensities. ARNON *et al.* gave no information from which rates or light intensities could be deduced.

A simple mechanism of phosphorylation—the creation of a high energy bond by the oxidation or reduction of an organic substance—would in all probability require the transfer of two electrons (or two hydrogens) for each ATP formed. The experimental value of 2.8 in Table VI, perhaps adjusted slightly downward to allow for some competition from the uncoupled system and also to allow for less than perfect recovery of the ATP formed, certainly points to the theoretical value of 2.0. However, we must beware of accepting too uncritically the implications of this convergence of the data from several laboratories on the gratifying value of 2.0; evaluation of the competition between the phosphorylating and the non-phosphorylating systems is still so uncertain and estimates of efficiencies are consequently so ambiguous that the postulated stoichiometry of the phosphorylation reaction could be quite wrong.

DISCUSSION

It has long been apparent that photosynthesis must consist, basically, of two processes, (a) the fixation of carbon dioxide in some organic substance, presumably to form a carboxyl group, and (b) the reduction of this carboxyl to the aldehyde level by a reductant formed in the photochemical dissociation of water. Unfortunately the direct reduction of carboxyl groups by any known biological reductant is a thermodynamic impossibility; when carboxyls are reduced in respiratory systems the combined action of pyridine nucleotides and ATP is required. It was necessary to suppose, therefore, either that the photosynthetic reductant formed *in vivo* by illuminated chloroplasts was an unknown substance of exceptional reducing potential or that the carboxyl reduction was accomplished in a coupled reaction analogous to the reactions in the respiratory system. When radioactive carbon dioxide was used to trace the "path of carbon" in photosynthesis, it was found[10] that the photosynthetic intermediates were, by and large, the intermediates of respiration. The participation of reduced pyridine nucleotides accordingly became more and more probable. Meanwhile a number of workers were able to show that illuminated chloroplasts could in fact reduce pyridine nucleotides, especially TPN^+[11-13]. SAN PIETRO AND LANG[13] were even able to obtain a protein from chloroplasts which catalysed the reduction, photosynthetic pyridine nucleotide reductase. Since the involvement of the pyridine nucleotides in photosynthesis seemed very probable, the question of the origin of the associated ATP was raised. At least one author[14] suggested that the ATP might be formed by the phosphorylation reactions of respiration using the primary photosynthetic reductant as electron donor. However, such a mechanism should result in an appreciable increase in oxygen uptake, an increase which does not seem to occur[15]. The problem was resolved independently by ARNON *et al.*[16] and FRENKEL[17] when they found a light-induced, oxygen-independent formation of ATP in *in vitro* preparations from photosynthetic organisms. This process has come to be called "photosynthetic phosphorylation" or "photophosphorylation".

The mechanism of photosynthetic phosphorylation has been the subject of much speculation. ARNON[18] originally suggested that the process was similar, in its essentials, to the process of oxidative phosphorylation in respiration, perhaps differing therefrom only in that precursors of the photosynthetically produced oxygen replaced molecular oxygen as the ultimate electron acceptor. He was led to this conclusion by the fact that some substances involved in the respiratory chain, for example FMN, seemed to be necessary for photosynthetic phosphorylation. However, there were objections to the concept. The requirement for FMN could be satisfied by other substances, such as phenazine methosulfate, which certainly are not intermediates in respiration. Moreover the mechanism proposed represented a somewhat uneconomic back-reaction which would necessarily lower the overall quantum efficiency of photosynthesis below the efficiency of the HILL reaction. Since the data of WAYRYNEN[19] and unpublished experiments by the author have shown that the quantum efficiency of photosynthesis and the HILL reaction are actually very nearly the same, it seems improbable that large amounts of ATP could be produced by this means. Whether we accept the conservative American estimates of quantum yields[20–22] or the more optimistic estimates of WARBURG AND NEGELEIN[23], photosynthesis is so efficient that we must look upon any mechanism which is inherently wasteful as of doubtful importance.

More recently ARNON and his colleagues[5] have produced evidence which suggests that 1 mole of ATP is formed during the reduction of 1 mole of TPN^+ (or 2 moles of ferricyanide). These observations have been confirmed by AVRON *et al.*[4]. If one assumes that each quantum of the red light absorbed by chlorophyll results in the photolysis of one molecule of water, the two quanta required for the reduction of a molecule of TPN^+ should yield more than enough usable chemical energy to form simultaneously a molecule of ATP. Consequently the new notion that the reduction of substances by chloroplasts may be directly linked with the phosphorylation of ADP seems more plausible in terms of efficient energy utilization than does the earlier concept of a phosphorylation-linked back-reaction of the products of the photolysis of water—the "cyclic phosphorylation" of ARNON. The discovery[3] that a large part of the electron transport in the HILL reaction depends on simultaneous phosphorylation and the observation of the converse dependence of phosphorylation on oxidant reduction both support the emerging picture of a phosphorylation-coupled HILL reaction so strongly that one is inclined to wonder if the phenomenon of "cyclic phosphorylation" is not an artifact caused by the spontaneous reoxidation of autoxidizable intermediates such as $FMNH_2$. In any case, it is no longer possible to doubt that chloroplasts can phosphorylate ADP in the course of reducing oxidants in general. The fact that the phosphorylation-independent pathway for the reduction of ferricyanide competes unfavorably with the phosphorylating pathway, and the fact that there is apparently no phosphorylation-independent pathway for the reduction of flavins, suggest that this "basic" system also may be unimportant *in vivo*. Perhaps it too represents some artifact arising from the use of such powerful oxidants as ferricyanide or such effective protein denaturants as quinones.

The manner in which amines permit electron transfer while by-passing the associated phosphorylation can only be guessed. Lipid solubility of the amine seems to be a requisite for activity, since the more polar polyfunctional amines and the ionized forms of the monofunctional amines are all inactive. Consequently the site of action of the amines is probably enclosed in and permeated by lipoprotein—a not surprising observation when we consider the known composition of chloroplasts[24]. Primary, secondary and tertiary amines are all "uncouplers" or activators, but they do not seem to be consumed in whatever reactions they activate. Any reactions entered into by the amines therefore must be reversible. However, the only reversible reaction which is common to these three classes of amines is probably the process of taking on a proton to become a positive ion. It would seem therefore that the function of amines must be related to this ability to ionize. The author would like to suggest, very tentatively, that the amines may simply be acting as bases which can penetrate to sites not available to highly polar bases, thereby causing hydroxyl ion catalyzed cleavage of high energy bonds which would otherwise transfer their energy to the pyrophosphate bond of ATP.

Let us suppose that phosphorylation is achieved by the following mechanism, which incidentally predicts the transfer of two electrons for each ATP formed:
overall:

$$\mathrm{X} + \mathrm{H_2O} \xrightarrow[\text{chloroplasts}]{\text{light}} \mathrm{XH_2} + \tfrac{1}{2}\mathrm{O_2}$$

$$\mathrm{XH_2} + \text{enzyme} \rightarrow \mathrm{XH_2}\text{-enzyme}$$

$$\mathrm{XH_2}\text{-enzyme} + \text{oxidant} \rightarrow \mathrm{X}{\sim}\text{enzyme} + \text{reduced oxidant}$$

$$\mathrm{X}{\sim}\text{enzyme} + \text{orthophosphate} \rightarrow \mathrm{X}{\sim}\text{phosphate} + \text{enzyme}$$

$$\mathrm{X}{\sim}\text{phosphate} + \mathrm{ADP} \rightarrow \mathrm{X} + \mathrm{ATP}$$

$$\text{oxidant} + \mathrm{H_2O} + \mathrm{ADP} + \text{orthophosphate} \xrightarrow[\text{chloroplasts}]{\text{light}} \text{reduced oxidant} + \mathrm{ATP} + \tfrac{1}{2}\mathrm{O_2}$$

In this reaction-sequence the omission of either ADP or phosphate would prevent the reduction of oxidant and the formation of oxygen. However, the high energy bond in X ~ enzyme very probably would be labile, since a "high energy bond" is by definition a bond which can be broken with the expenditure of little energy. Consequently it is not unreasonable to suppose that hydroxyl ions associated with amines might catalyse the hydrolysis of such a bond, in which case the dependence of the system on phosphorylation would disappear. On the other hand, the direct reaction of strong oxidants with $\mathrm{XH_2}$ might result in a phosphorylation-independent, amine-insensitive electron transfer, the "basic" system which, heretofore, has been the object of most HILL reaction studies.

The phosphorylation mechanism outlined above is analogous to the substrate level phosphorylations of respiration. It is, in fact, taken directly from the postulated mechanism of triosephosphate dehydrogenation. There are several reasons for relating the HILL reaction phosphorylations to substrate phosphorylation rather than to respiratory chain phosphorylations: (a) The apparent stoichiometry of two electrons transferred for each ATP formed coincides with the stoichiometry of the single stage phosphorylation-coupled exidation of substrates and not with the multistage oxidations through the respiratory chain. (b) HILL reaction phosphorylations may be associated with the reduction of pyridine nucleotides. Consequently XH_2 must be an electron donor of reducing potential comparable to triosephosphate or α-ketoglutarate. (c) HILL reaction phosphorylations, like substrate phosphorylations, are not uncoupled from the associated electron transfers by 2,4-dinitrophenol. However, this analogy has limits; the author is not aware of any evidence which suggests that ammonia or amines affect the phosphorylations associated with the oxidation of α-ketoglutarate or triosephosphate.

ACKNOWLEDGEMENTS

The author wishes to take this opportunity of expressing his gratitude to Dr. R. HILL, in whose laboratory this work was begun, for his unfailing encouragement. Thanks are also due to Dr. A. JAGENDORF for his helpful comments and for the release of manuscripts of papers in the press. The earliest phase of this investigation was supported by the Agricultural Research Council of Great Britain.

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PURIFICATION OF STAPHYLOCOCCAL COAGULASE

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(Received October 13th, 1959)

SUMMARY

A method is described for isolating staphylococcal coagulase. Fractionations are carried out with ammonium and cadmium sulfates at various pH. The partially purified enzyme is then subjected to chromatographic procedures employing calcium phosphate. A material which migrates as a single component under the conditions of electrophoresis is described. The coagulase activity of this enzyme is greater than any previously described.

INTRODUCTION

In 1935 WALSTON[1] demonstrated that cell free filtrates of *Staphylococcus aureus* are capable of coagulating oxalated plasma. Subsequent attempts at purification have met with only partial success[2,3]. Our present work has led to the isolation of this type of coagulase which has more activity than the best previously reported preparations[4]. The physical properties of the present material indicate, moreover, greater homogenity than those of previous investigations.

MATERIALS AND METHODS

Previous workers have utilized serum[5] and tissue infusion enriched media for the growth of staphylococci in order to obtain maximum yields of coagulase. We have preferred a liquid peptone medium, because it lends itself more readily to purification procedures, even though the enzyme yield is less. Tryptose broth (Difco) was used exclusively for growing *Staphylococcus aureus* (TAGER's Strain[6]) and was prepared in a concentration of 26 g of dry powder/l. This was enriched with crystalline thiamine (5 mg/l) and the pH adjusted to 7.2–7.6. 15 l of broth were inoculated with actively growing staphylococci (in broth) and incubated at 37° for 72 h with occasional agitation. A crude enzyme preparation was made by fractionating the medium with ammonium and cadmium sulfates (see flow sheet).

The crude enzyme was then dialyzed against 0.001 *M* phosphate buffer, pH 6.8. It was then passed over a calcium phosphate hydroxyapatite chromatographic column prepared according to the method of TISELIUS[7]. The column was washed in the cold with 0.001 *M* phosphate buffer steps in increasing increments of 0.02 *M*. Each

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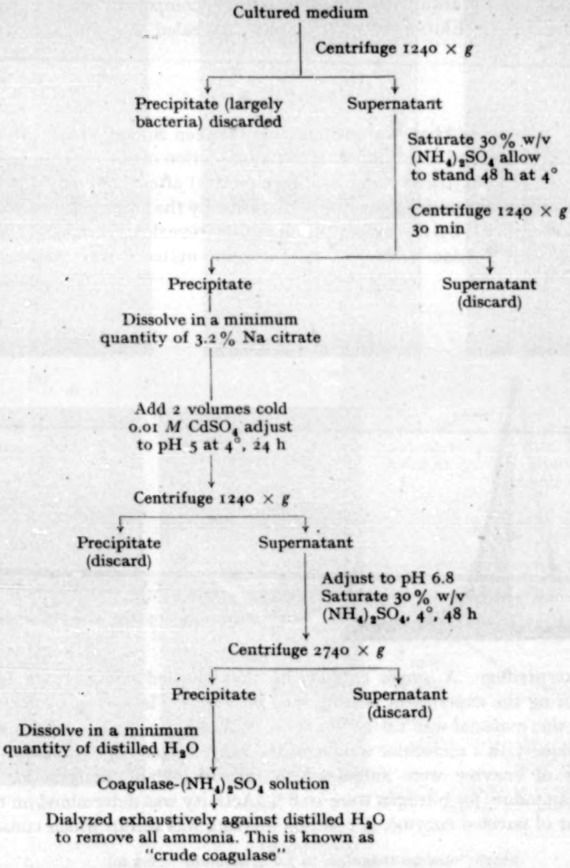
FLOW SHEET


Cultured medium
Centrifuge 1240 × g
Precipitate (largely bacteria) discarded
Supernatant
Saturate 30% w/v $(NH_4)_2SO_4$ allow to stand 48 h at 4°
Centrifuge 1240 × g 30 min
Precipitate
Supernatant (discard)
Dissolve in a minimum quantity of 3.2% Na citrate
Add 2 volumes cold 0.01 M $CdSO_4$ adjust to pH 5 at 4°, 24 h
Centrifuge 1240 × g
Precipitate (discard)
Supernatant
Adjust to pH 6.8 Saturate 30% w/v $(NH_4)_2SO_4$, 4°, 48 h
Centrifuge 2740 × g
Precipitate
Supernatant (discard)
Dissolve in a minimum quantity of distilled H_2O
Coagulase-$(NH_4)_2SO_4$ solution
Dialyzed exhaustively against distilled H_2O to remove all ammonia. This is known as "crude coagulase"

elution was accomplished by allowing a volume of buffer equal to two times the volume of hydroxyapatite adsorbent to flow through. Each eluate fraction was tested for coagulase activity by incubating with stored human plasma. The coagulase activity was confined to one or two steps of elution. The point of coagulase elution was determined for each batch of hydroxyapatite.

The fraction containing the coagulase activity was dialyzed exhaustively against distilled water. This solution was then lyophilized. A sample of the lyophilized material was subjected to paper electrophoresis in barbital buffer at pH 8.6. If two components were present, the material was redissolved in phosphate buffer and rechromatographed through a fresh hydroxyapatite column. The active fraction obtained from the adsorption and elution always consisted of a single component when examined by paper electrophoresis. Elution of this material revealed uniform activity in the activation of fibrinogen.

RESULTS

The average yield per 15 l batch of medium was between 20 and 30 mg of lyophilized material. This represented a two-hundred fold purification of the enzyme on the basis of dry weight of protein (trichloractic acid precipitable) after the removal of bacteria.

Examination of the purified enzyme preparation by the moving boundary method in 0.1 ionic strength, pH 8.6 diethylbarbiturate buffer revealed a component migrating at $-1.1 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1} (see Fig. 1). The same material was examined in the Spinco ultracentrifuge. A single component that showed considerable boundary spreading during the experiment is evidenced by Fig. 2. The average sedimentation constant for this material was $1.0 \cdot 10^{-13}$ cm sec^{-1}. Assuming a spherical shape for the molecules in question a molecular weight in the range of 5000 is obtained.

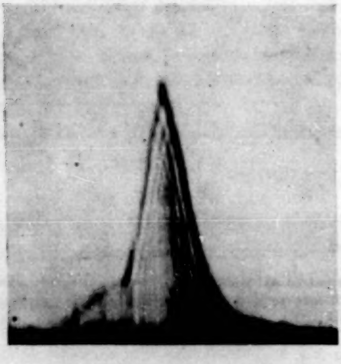
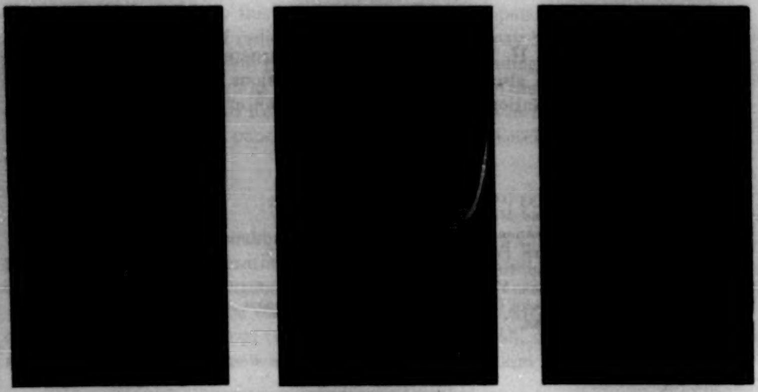


Fig. 1. Descending electrophoretic boundary of enzyme in pH 8.6 veronal buffer. Duration of experiment was 113 min at a potential gradient of 4.5 V/cm.

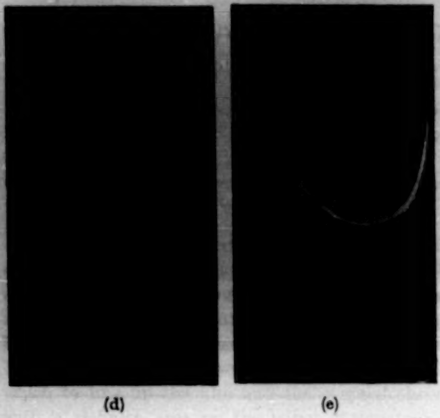
Samples of enzyme were subjected to micro-Kjeldahl analysis for percent nitrogen. Mean values for nitrogen were 16.8 %. Activity was determined on the basis of dry weight of purified enzyme. A reaction mixture was set up which consisted of:

Staphylococcus coagulase in 3.0 % albumin	0.5 ml
Fibrinogen (Bovine) 1.0 %	0.5 ml
Human plasma 1:100 (H_2O)	0.1 ml
	1.1 ml

The diluted plasma was added to supply the minimal quantity of coagulase reacting factor[5,8] necessary to complete the reaction rapidly. The reaction mixture was incubated at 37°. The end point used was the time necessary to form a complete clot which was resistant to agitation and would allow inversion of the tube. At a concentration of 0.1 µg of enzyme a complete clot was observed within 1000 min (see Fig. 3). At a concentration of 0.01 µg of enzyme a clot was observed, but did not occupy all of the reaction mixture.



(a) (b) (c)



(d) (e)

Fig. 2. Ultracentrifuge pattern of enzyme in 0.2 ionic strength, pH 7.4 potassium phosphate buffer. Exposures at 32 min intervals at 59,380 rev./min.

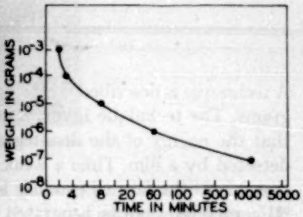


Fig. 3. Activity of Staphylococcus coagulase per unit dry wt.

DISCUSSION

Although two criteria for purity have been satisfied (electrophoresis and ultracentrifuge data) insufficient evidence is presented here for a guarantee of purity. The boundary spreading seen in the ultracentrifuge can be explained by the small molecular size of the enzyme and subsequent diffusion. However, an alternate explanation is the presence of a large family of molecules of similar size. The small molecular size of the enzyme (smaller than any other enzyme previously reported) raises the question as to whether the isolated substance represents an active degradation product of the enzyme originally manufactured by the cell. This is plausible in view of the proteolytic enzymes present in a medium which contains a large population of cells. If this were so, variation in molecular size might easily be expected.

ACKNOWLEDGEMENTS

We are indebted to Dr. H. Deutsch for the data garnered by electrophoresis and ultracentrifuge. We are also grateful for his suggestions and advice. One of the authors (M. M.) was a National Institute of Health Cancer Trainee.

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Biochim. Biophys. Acta, 40 (1960) 518–522

DETECTION OF TRITIUM ON PAPER CHROMATOGRAMS*

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(Received October 6th, 1959)

SUMMARY

A technique is described whereby ^{3}H compounds may be detected on paper chromatograms. The technique involves soaking the chromatogram in a liquid scintillator so that the energy of the disintegrating tritium atoms is converted into light which is detected by a film. Thus a weak β-particle is converted into light quanta which can travel into the film to produce an image. By the use of fast film, small amounts of ^{3}H compounds can be separated and identified in only a few days.

INTRODUCTION

Hydrogen is an element of fundamental importance in biological processes. It is therefore important to have a method for studying its pathway through living systems. Paper chromatography[2] is an extremely powerful technique for the separation of complicated mixtures of compounds and has found much application in the field of biochemistry. One of the greatest problems of paper chromatography is the detection of the individual compounds on the paper. This has been done by spraying the paper with suitable chemicals which develop colour reactions with the compounds

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* A brief account of this technique has been published[1].
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Biochim. Biophys. Acta, 40 (1960) 522–526

of interest. Unfortunately this usually destroys the compounds and is not of general application. In the case of radioactive materials a film may be laid on the chromatogram[3] which on development gives dark areas corresponding to the radioactive compounds. This technique[3] works well for ^{14}C, and ^{32}P, but in the case of ^{3}H the maximum energy of the β-particle (18 KeV) is too low for it to leave the paper and enter the emulsion of the film. This paper describes a technique whereby this difficulty is overcome.

DESCRIPTION OF METHOD

The technique involves soaking the chromatogram in a liquid scintillator so that the energy of the disintegrating tritium atoms is converted into light quanta which can travel into the film to produce an image.

In actual practice the chromatogram is attached, with wire staples, to some convenient backing material (used X-ray film is suitable). Marker segments, which are pieces of filter paper on which have been placed tritium labelled compounds (see below for more details), are attached to the corners of the backing sheet with staples. These later provide reference points on the backing sheet and also on the film with which to line up the film and the original chromatogram, thus identifying a spot with a definite area on the paper chromatogram. The chromatogram sheet thus prepared is laid in the bottom of a shallow tank and covered with a scintillating liquid (see below for discussion of scintillation liquids). A sheet of screen type X-ray film (see below for discussion of film) is next placed on top of the chromatogram, taking care to eliminate any bubbles. The tank is then covered with an air-tight top. After a few days, the film is removed from the tank, drained of scintillating liquid and allowed to dry in the air of the dark room. When all the toluene has drained or evaporated from the film, it is placed in the wash water and wiped with a soft cloth to remove any terphenyl which may have remained. The film is then developed and fixed in the normal manner. Such a "scintillation autogram" is shown in Fig. 1. Thus, as in ^{14}C radioautography, compounds can be tentatively identified by their position and this identification confirmed by removal from the paper and co-chromatography with known compounds, or by chemical treatment and re-chromatography.

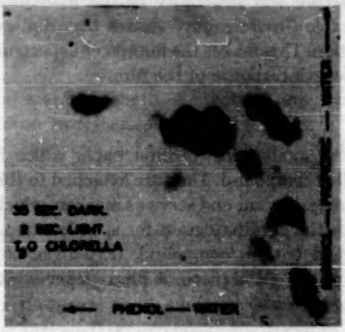

Fig. 1. "Scintillation autogram". Explanation see text.

DETAILS OF METHOD

Apparatus

The author's tanks are made by soldering 1/4″ square extruded brass rod onto a heavy gauge brass sheet of suitable size taking care to keep the top as flat as possible. Another piece of brass plate serves as a lid which is kept in place with a lead brick. The tanks are kept in a dark-room cupboard.

Scintillation liquid

The classical scintillation solution viz: 3 g/l *p*-diphenyl benzene (terphenyl) in toluene which is used for scintillation counting is quite satisfactory. The presence of sulphur compounds in toluene may cause some quenching of the light but this is a relatively small effect and can be overcome by the use of "sulphur free" toluene which is commercially available, or by passing the toluene down an alumina column. Since commercial medical "screen type" X-ray film is made to be used with a calcium tungstate fluorescent screen the sensitivity of the method can be improved some 40% by adding a suitable "wavelength" shifter (*e.g.* diphenyl heaxtriene 0.01 g/l) to the scintillating solution. This makes the fluorescent spectrum of the solution match more closely the wavelength response of the film.

Marker segments

Marker segments are small pieces of filter paper which have been impregnated with some tritium labelled compound. They are attached to the corners of the backing sheet along with the chromatogram and serve as markers which enables the developed film to be lined up with the chromatogram for spot location. They are prepared by placing a spot of labelled tritium compound, (*e.g.* algae or yeast which has been treated with tritiated water) on a piece of filter paper which, after drying, is cut into segments.

Film

For maximum sensitivity it is advantageous to have as fast a film as possible. For practical purposes it is also an advantage to have the film in reasonably large sheets. The manufacturers of film for normal photographic purposes sacrifice speed (even in the very fast films) in order to keep the grain size down. Screen type X-ray film on the other hand is much faster to light than even the fastest of normal photographic films. That it has a large grain size is not important in this application. Screen type X-ray film (such as Kodak "Blue Band") has the added advantage that it is readily available in large (14″ × 17″) sheets which are used for conventional chest x-rays. It also can be used with a safety light since it is relatively insensitive to light of the longer wavelengths.

Film processing

The developer and fixer as supplied by the film manufacturers when used according to their directions will produce the optimum image. Care should be taken, however, to dip the film in water and wipe it with a soft wet cloth to remove any terphenyl before placing in the developer.

Exposure time and application to ^{14}C chromatograms

A question that immediately presents itself is whether this technique can be applied to very low level ^{14}C chromatograms which take a very long time to expose in the normal manner. Unfortunately this technique is of no help in this problem. The technique is more sensitive for short exposures (< 24 h) than is conventional radioautography, but for longer exposures it is progressively less sensitive. This is in the nature of the photographic process itself. In conventional radioautography one is developing the actual β-particle tracks and the intensity of these is to a first approximation independent of the exposure time. In the case of the photography of light, however, which is the principle of the scintillation autography technique, there exists for a given film an optimum time during which a given quantity of light will produce the greatest effect. This period is much less than a day for any film. Thus for longer exposures the effect of a given quantity of light decreases as the length of time over which it is received increases. Thus, if long exposures (many weeks) are necessary for weak ^{14}C chromatograms then conventional radioautography is the best approach. Similarly for tritium experiments enough isotope should be used to get the desired exposure in less than a week.

Sensitivity

This of course depends on the length of exposure time, but if 3 g/l terphenyl and 0.01 g/l diphenyl hexatriene in toluene are used with Kodak "Blue Band" film then a spot whose area is one square cm, and whose activity is 0.1 μC can be easily detected after an exposure of 50 h.

DISCUSSION

An example of a scintillation autograph is shown in Fig. 1. As can be seen the quality and sharpness compares favourably with radioautograms produced by the β-particles from ^{14}C. Because of the universal occurrence of hydrogen in biological systems, and the availability, low health hazard and cheapness of tritium (10^{-4} that of ^{14}C) coupled with the ease of preparation of ^{3}H compounds, this technique should find wide application.

Tritium can be introduced easily into many organic molecules, either by hydrogenation of the corresponding unsaturated compound or by exposure to tritium gas[4]. This latter method of preparation (The Wilzbach synthesis) seems to be of a very general application, but suffers from the fact that it yields a product contaminated with small amounts of ^{3}H material of high specific activity. With the technique described in this paper, however, it is possible to prepare chromatographically pure ^{3}H compounds. CRAWFORD[5] has exposed a mixture of amino acids to tritium gas and separated the resulting mixture using paper chromatography and the above technique.

ACKNOWLEDGEMENTS

The author wishes to thank W. H. **Melhuish** of the Institute of Nuclear Science and N. J. **Rumsey** of the Dominion Physical Laboratory, for helpful discussions and the former for the absolute determination of the tritium in chromatogram spots.

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Biochim. Biophys. Acta, 40 (1960) 522–526

VITAMIN D AND THE STRUCTURE OF KIDNEY MITOCHONDRIA*

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(Received October 23rd, 1959)

SUMMARY

An electron microscopic examination has been made of thin sections of kidney mitochondria isolated in 0.44 *M* sucrose from vitamin D deficient rats and from vitamin D treated rats. Vitamin D deficiency resulted in swollen and morphologically damaged mitochondria characterized by large intercristal spaces and disrupted cristal systems. These changes were prevented by vitamin D.

INTRODUCTION

The well-established accumulation of citrate in tissues following vitamin D administration[1] prompted our investigation of Krebs cycle oxidations in vitamin D treated and deficient rats[2]. It was found that vitamin D specifically reduced the oxidation of citrate and isocitrate by kidney homogenates and mitochondria[2,3]. This effect was also produced by the addition of vitamin D *in vitro*[4]. Further studies[5] to be published in detail later, suggested that the reduction in oxidation was not necessarily due to an enzymic inhibition, but was possibly due to physical inhibition of citrate penetration of the mitochondria. In support of this, it has now been found that the morphology of mitochondria isolated from vitamin D deficient rats differs markedly from that of vitamin D supplemented rats.

METHODS

Young, male Holtzman rats weighing 70–80 g were fed *ad libitum* an adequate Ca and P semisynthetic diet with and without vitamin D for 21–28 days as described earlier[1,2]. They were killed by a sharp blow on the head followed by decapitation. The kidneys

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* Published with the approval of the director of the Wisconsin Agricultural Experiment Station. We are indebted to the Wisconsin Alumni Research Foundation for financial support.

Biochim. Biophys. Acta, 40 (1960) 526–530

were quickly immersed in ice-cold 0.44 *M* sucrose solution. A 10 % homogenate of the chilled tissues in 0.44 *M* sucrose was prepared with a Potter-Elvehjem homogenizer fitted with a Teflon pestle (A. H. Thomas Co., U.S.A.) and 10 ml of the homogenate was layered over 10 ml of 0.52 *M* sucrose in a lusteroid tube at 0°. The layered preparation was centrifuged in an International PR-2 refrigerated centrifuge equipped with a high speed rotor No. 296 at 800 × *g* for 12 min to remove nuclei, cellular debris, unbroken cells, etc. The upper layer was centrifuged at 3500 × *g* for 12 min to sediment the mitochondria. The resulting pellet, which was washed once with 0.44 *M* sucrose was a relatively pure mitochondrial preparation, as was revealed by both electron and phase-contrast microscopy. This method was a modification of the method used for the preparation of liver mitochondria in 0.44 *M* sucrose[6], since with kidney tissue that technique gave poor yields and impure preparations.

The mitochondria were fixed for 2 h in a buffered, 2 % osmium tetroxide-hypertonic sucrose solution (0.16 *M* veronal–acetate buffer, pH 7.2, 0.60 *M* sucrose, 0.08 *M* osmium tetroxide), essentially according to the method of PALADE[7]. The fixed pellet was broken up and washed 3 times with 0.32 *M* veronal–acetate buffer, pH 7.2. It was dehydrated by washing consecutively with ethyl alcohol of gradually increasing concentrations and finally with butyl methacrylate. Samples of the pellet were embedded at 49° for 36–48 h in a mixture of butyl methacrylate–methyl methacrylate (4:1) with 0.8 % benzoyl peroxide as the catalyst. Sections were cut with a Servall Porter-Blum microtome, and examined with an RCA EMU-2 or a Siemens Elmiscop I electron microscope.

RESULTS AND DISCUSSION

Preliminary observations with a phase-contrast microscope revealed that kidney mitochondria from vitamin D deficient rats were larger than those isolated from corresponding rats which had received vitamin D. Furthermore they indicated differences in structure. These indications have been confirmed and extended by observations with the electron microscopes on preparations made in parallel from vitamin D treated and vitamin D deficient rats.

In the vitamin D deficient rats there was a consistent increase and a greater variation in the size of mitochondria. Table I presents data obtained by measuring all the mitochondria in the central area of comparable electron micrographs. At least 25 mitochondria were measured for each experiment without selective elimination. Figs. 1 and 2 also show the difference in size and in addition a difference in structure. The preparations from the deficient rats are characterized by large intercristal spaces or vacuoles (C). In many cases the cristae are withdrawn from the outer mitochondrial membrane (E). Fragmentation or disruption of the cristal organization occurs with some degree of frequency (D). In most cases, however, the outer double membranes and cristae are still present.

By way of contrast, the mitochondria from the vitamin D treated rats are relatively intact structures (Fig. 2). The outer double membrane closely envelops a tightly and uniformly arranged cristal system in which few intercristal vacuoles appear. Disrupted cristal systems or cristae withdrawn from the outer membrane are rarely encountered.

To our knowledge, electron micrographs of isolated kidney mitochondria have

TABLE I

THE EFFECT OF VITAMIN D ON THE DIMENSIONS OF ISOLATED KIDNEY MITOCHONDRIA

The rats were fed an adequate Ca and P containing diet for 21–28 days. Where indicated the rats received 75 I.U. of vitamin D every 3 days. Each figure represents an average measurement of 25 mitochondria from a given experiment.

Group	*Expt. No.*	*Greatest diameter* μ	*Smallest diameter* μ
No vitamin D	1	0.91 ± 0.25*	0.47 ± 0.12*
	2	0.98 ± 0.25	0.58 ± 0.21
	3	1.00 ± 0.31	0.61 ± 0.15
	4	1.05 ± 0.36	0.51 ± 0.12
	5	0.95 ± 0.36	0.52 ± 0.18
Average		0.98 ± 0.31	0.54 ± 0.16
Plus vitamin D	1	0.62 ± 0.18	0.39 ± 0.09
	2	0.85 ± 0.19	0.61 ± 0.12
	3	0.77 ± 0.18	0.43 ± 0.10
	4	0.84 ± 0.22	0.51 ± 0.12
	5	0.87 ± 0.18	0.51 ± 0.09
Average		0.79 ± 0.19	0.49 ± 0.10

* Standard deviation = $\sqrt{\Sigma d^2/n}$.

not yet appeared in the literature, although SJÖSTRAN DAND RHODIN[8] and PALADE[9] have published micrographs of kidney mitochondria *in situ*. On the other hand, numerous micrographs of liver and heart mitochondria, both isolated and *in situ*, have been published[6,8–10]. WITTER *et al.*[6], found that liver mitochondria isolated in 0.44 *M* sucrose solutions closely approached the morphology of mitochondria in intact tissue. ZIEGLER *et al.*[10], also showed that the use of hypertonic sucrose solutions resulted in the isolation of nearly intact structures. Similarly, the isolation of kidney mitochondria (Fig. 2) from 0.44 *M* sucrose solutions yields relatively intact mitochondria as judged by their similarity to *in situ* micrographs[8].

The now abundant morphological studies on isolated mitochondria opens the opportunity for a correlation of structure with the ability to oxidize externally added citrate. It has been known[11] for some time that heart mitochondria cannot oxidize added citrate, but can oxidize internally generated citrate. This finding has been reaffirmed[12] recently and correlated with mitochondrial structure. It is also well established[9] that heart mitochondria contain a large number of cristae per unit of mitochondrial volume arranged in a tightly-knit branching system. In contrast to the heart particles, liver mitochondria readily oxidize external citrate and have comparatively few cristae which are widely separated from each other[9]. Furthermore, kidney mitochondria from normal rats (Fig. 2), which oxidize added citrate very slowly[5], approximate heart particles in their compact and dense cristal arrangement while those from vitamin D deficient rats, which oxidize citrate more rapidly, have more widely separated cristae (Fig. 1). From these spatial relations it might be permissible to assume that the citrate molecules are oxidized slowly because they have more difficulty in gaining access to the oxidative sites in the more densely packed particles. This is supported by the observation of ATP inhibition of citrate oxidation[5,11,13], since ATP is known to reverse or prevent mitochondrial swelling[14,17].

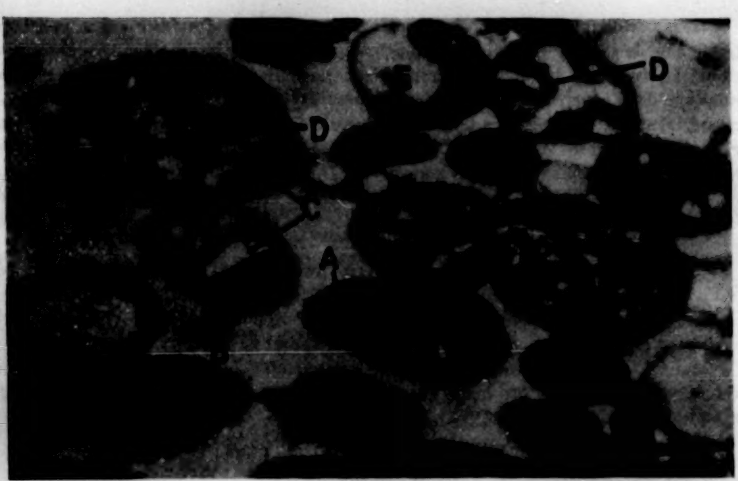

Fig. 1. Electron micrograph of kidney mitochondria isolated in 0.44 *M* sucrose from a vitamin D deficient rat (adequate Ca and P diet). The outer double membranes are clearly discernible (A), as are the cristae (B). Note: the large intercristal spaces (C); the swollen appearance of most mitochondria; the disrupted cristal arrangements (D), and the separation of the cristae from the outer membrane (E). Magnification: 34,000 ×.

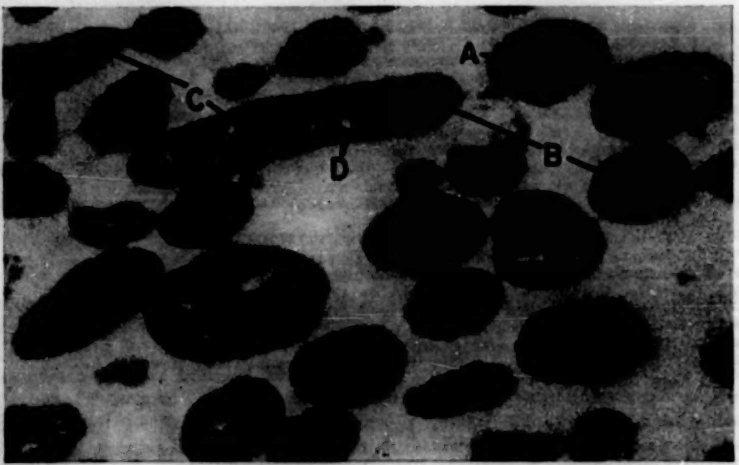

Fig. 2. Electron micrograph of kidney mitochondria isolated in 0.44 *M* sucrose from a vitamin D supplemented rat. (Adequate Ca and P diet plus 75 I. U. of vitamin D_2 every 3 days.) The outer double membrane (A) envelops a tightly packed and highly organized cristal system (B). Note the rods (C) and the small intercristal spaces (D) which are poorly visible in most cases. Magnification: 34,000 ×.

It is not certain that the morphological differences observed here occur in intact tissue as it is entirely possible that the deficiency in vitamin D so changes the mitochondria that they become susceptible to injury by the technique used for their isolation. This nevertheless means that vitamin D had effected some change in mitochondrial structure. There is a possibility that a difference in homogenate composition may also account for these changes, since numerous agents which promote or prevent mitochondrial swelling are now known[16,18]. This now appears unlikely, since suspending the mitochondria from vitamin D treated rats in the supernatant fraction from vitamin D deficient homogenates and vice versa resulted in no change in citrate oxidation.

Because of the intimate relation between vitamin D and Ca metabolism, the role of Ca and other cations should be considered with regard to mitochondrial damage. On the basis of our present knowledge it does not appear that these changes could have been effected through Ca, since vitamin D would be expected to elevate kidney Ca[2] and Ca is a known swelling agent for isolated mitochondria[16]. These studies on mitochondrial structure are being extended to give a more thorough understanding of factors affecting morphology.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Dr. H. HIFT for her generous help in the phase-contrast microscope work and to S. SACHS for her invaluable sectioning work.

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Short Communications

A mild procedure for separating enzymes and cofactors

The successful use of the polydextran Sephadex[1] in separating large molecules from small molecules indicated that this material might be useful in the separation of enzymes from cofactors. The present report illustrates an instance of such a separation. The enzymes involved were obtained from a water extract of acetone-dried *Escherichia coli* PA15, a mutant which requires either serine or glycine for growth[2]. The organism was grown on minimal medium plus glycine[3] and the extracts were prepared as described[3]. Such extracts can synthesize methionine from homocysteine and serine when supplemented as indicated in Table I. In addition a coenzyme form of folic acid found in heated cell extracts is required[4,5].

TABLE I

THE RESTORATION OF METHIONINE SYNTHESIS IN SEPHADEX-TREATED AND DIALYSED ENZYME EXTRACTS BY COFACTOR ELUTED FROM SEPHADEX

Each incubation mixture contained 4.6 mg protein, 1 μmole diphosphopyridine nucleotide, 10 μmoles adenosine triphosphate, 10 μmoles fructose 1,6-diphosphate, 1 μmole pyridoxal phosphate, 10 μmoles L-serine, 10 μmoles $MgSO_4$, 20 μmoles DL-homocysteine, 100 μmoles potassium phosphate, pH 7.8. Total volume 2 ml. Incubated 3 h at 37° under H_2 in Thunberg tubes. Methionine was determined microbiologically[7] with *Leuconostoc mesenteroides* P60 (ATCC 8042). Methionine values are corrected for the small amount of methionine present in the eluted cofactor preparation (0.02 μmole methionine in 0.1 ml).

Enzyme preparation	*Addition*	*Methionine (μmole)*
Untreated	None	0.71
Sephadex-treated	None	0.00
Sephadex-treated	0.1 ml eluted cofactor	0.65
Sephadex-treated	0.2 ml eluted cofactor	0.83
Sephadex-treated	0.4 ml eluted cofactor	0.84
Dialysed	None	0.38
Dialysed	0.1 ml eluted cofactor	1.00
Dialysed	0.2 ml eluted cofactor	0.95

A 2 × 12 cm Sephadex G-50* column was prepared as described[1] and washed with 100 ml 0.01 *M* potassium phosphate buffer, pH 7.8 and chilled to 5° in the cold room where subsequent elution was carried out. A 10-ml portion of an extract of acetone-dried cells containing 7.8 mg protein/ml[6] was pipetted onto the column and 5-ml fractions were collected using the above-mentioned buffer as eluant. 95 % of the added protein was recovered in combined fractions 2, 3 and 4. A distinct yellow band could be seen remaining on the column. Continued elution with the same buffer removed this band. Fractions showing an absorbancy (Beckman DU Spectrophotometer, 1-cm light path) at 260 mμ greater than 1.0 (fractions 8 through 14) were combined and lyophilized. The elution of enzyme and cofactor was complete in 30 min.

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* Obtained from Pharmacia, Uppsala, Sweden or Pharmacia Laboratories Inc., 501 Fifth Avenue, New York 17, N. Y., U.S.A.

The lyophilized material (total dry wt. 30 mg excluding buffer) was taken up in 2 ml water and tested for its ability to reactivate depleted enzyme preparations (Table I). The cofactor solution showed an absorption peak at 260 mμ with a ratio of absorption at 280 mμ to 260 mμ of 0.43.

A second 10-ml portion of the extract of acetone-dried cells was enclosed in Visking cellulose casing (8/32 inches). The casing was tied to a mechanical stirrer and suspended in 4 l of the above-mentioned buffer. Stirring was continued for 20 h at 5°.

The results shown in Table I indicate that the folic acid coenzyme was completely removed by the Sephadex treatment whereas the dialysed preparation still retained 54 % of the activity of the untreated extract. 92 % of the activity could be restored to the Sephadex treated extract by adding the amount of cofactor preparation (0.1 ml) derived from 0.5 ml of the untreated enzyme extract. Doubling the amount of the cofactor preparation increased the activity to 117 % that of the untreated extract. Further addition of cofactor did not result in increased methionine synthesis. The eluted cofactors also restored activity to the partially depleted dialysed extract.

This procedure for separating enzymes and soluble cofactors is more satisfactory than dialysis in that it is more rapid, cofactors are removed more completely and the cofactors are obtained in a relatively small volume.

I would like to thank Dr. E. Huff for calling my attention to Sephadex and Mrs. M. Romine for performing the methionine assay.

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Received November 28th, 1959

The prosthetic group of a chromoprotein from mycobacteria

Several yellowish pigments soluble in aqueous media have been isolated from acid-fast bacteria, *e.g.*, phthiocol[1], riboflavin[2] and pterins[3,4].

During the isolation of a lactic acid oxidase from *Myco. smegmatis*[5] a yellow chromoprotein which accompanies the oxidase through several stages of purification was separated in a partly purified state.

This protein, which is not known to have enzymic activity, showed a characteristic absorption spectrum with maxima at 276 mμ and 403 mμ, the ratio $E_{403\,\mathrm{m}\mu}/E_{276\,\mathrm{m}\mu}$ being 1:3 for the purified preparation (Fig. 1). Acidified solutions of the protein are very pale yellow and this colour is intensified on making alkaline, the change being accompanied by increased absorption at 403 mμ. Treatment with HCl to give a final concentration of 0.1 *N* causes precipitation of the protein and release of the bound pigment.

As only small amounts of the chromoprotein were available the pigment was isolated from intact bacteria. The bacteria were extracted with acetone in a Soxhlet apparatus and dried *in vacuo*. Portions of the acetone powder (5–10 g) were dispersed in 200 ml 5 % (*w*/*v*) trichloracetic acid and shaken mechanically for 4 h. On removal of the cells the yellow extract was treated repeatedly with ether to remove the trichloroacetic acid and evaporated to a small volume in a lyophyliser. The concentrate was run on to a column of Florisil which effectively retained a number of the contaminating compounds. With 1 % acetic acid as solvent the yellow chromophore was present in the first of several fluorescent bands eluted from the column. This fraction was concentrated and run on a starch column using 0.01 *N* HCl as solvent. Again the pigment was present in the first fluorescent band eluted. Barium acetate was added to this eluate, the pH adjusted to 8 and ethanol added to a final concentration of 80 %. A yellow flocculent precipitate was centrifuged off, washed with 80 % ethanol and dried *in vacuo*. Further purification was obtained by re-solution of the precipitate in the minimal amount of 0.1 *N* HCl, precipitation of the barium as the sulphate and re-precipitation of the pigment as the barium salt.

The pigment can also be purified by countercurrent distribution with 1 *N* HCl saturated with ammonium sulphate as the stationary phase and *n*-butanol saturated with 1 *N* HCl as the mobile phase. After 180 transfers the pigment, readily detected by its fluorescence, was obtained in the aqueous phase of the first few units.

The absorption spectra in 0.1 *N* HCl and in 0.1 *N* NaOH are given in Fig. 2. The spectrum shows a marked bathochromic shift on change from acid to alkali, with absorption maxima at 265 mμ and 375 mμ in 0.1 *N* HCl and at 252 mμ, 268 mμ and 418 mμ in 0.1 *N* NaOH. The pigment dissociated from the chromoprotein appears

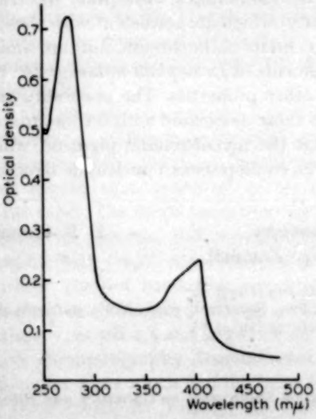

Fig. 1. Absorption spectrum in 0.05 *M* acetate buffer, pH 4.5, of the chromoprotein isolated from *Myco. smegmatis*.

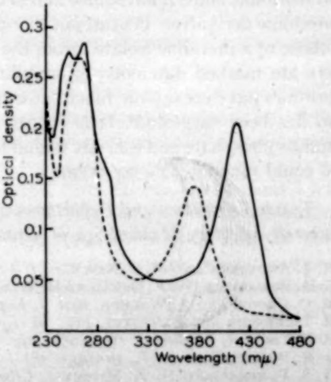

Fig. 2. Absorption spectrum of the yellow pigment isolated from acetone-treated cells of *Myco. smegmatis*. – – –, 0.1 *N* HCl; ———, 0.1 *N* NaOH.

to be identical with the pigment isolated from the intact bacteria as judged by similarity in absorption spectra, changes in fluorescence and behaviour on paper chromatograms with 5% Na_2HPO_4 (R_F, 0.64) and butanol–acetic acid–water (4:1:5) (R_F, 0.06) as solvents.

The pigment is strongly yellow coloured in neutral or alkaline solutions but the colour decreases on acidification or addition of $Na_2S_2O_4$. Under u.v. light solutions of the pigment show an intense yellow-green fluorescence below pH 2 and a blue fluorescence at higher pH values. This strong fluorescence and the colour change in fluorescence induced by alteration of pH afford a ready means of following the isolation of the pigment.

The pigment was insoluble in diethyl ether, light petroleum, chloroform, ethyl acetate, and *n*-butanol, sparingly soluble in cold water and ethanol, but readily soluble in dilute acids, salt solutions and liquid phenol, particularly on warming. The pigment showed remarkable resistance to acid hydrolysis (refluxing in 5 *N* HCl for 18 h) and towards mild oxidation with alkaline $KMnO_4$. Both treatments give rise to a number of fluorescent compounds detected by paper chromatography but the principal compound present showed the properties of the original pigment (R_F, colour change, fluorescence). After hydrolysis with $HClO_4$, purines and pyrimidines could not be detected by paper chromatography with isopropanol–2 *N* HCl as solvent[6].

An insoluble compound could be formed from the pigment by treatment of solutions with 0.01 *M* $FeCl_3$ at pH 5 and a barium salt of the pigment was precipitated at pH 8 by 80% ethanol in the presence of barium acetate. Although insufficient material was available for full elementary analysis, estimation of nitrogen (micro-Kjeldahl) on a sample of the free pigment showed the presence of 18% N and estimation of phosphorus on a sample of the Ba salt gave 5.2% P. A pigment with identical properties to those described was isolated by the same procedure from *Myco. Karlinski* and *Myco. phlei*.

The pigment described has certain properties (solubilities, absorption spectrum, bathochromic shift, fluorescence and acid stability) which are consistent with those of a pteridine derivative. Features of the spectrum and its bathochromic shift are similar to those of a pteridine isolated from the eye pigments of *Drosophila melanogaster*[7] but there are marked differences in stability and other properties. The possibility that pteridines have coenzymic functions other than those associated with tetrahydrofolic acid has been suggested[8]. It is conceivable that the mycobacterial pigment, which contains phosphate and is firmly bound to protein, could possess a nucleotide structure and could function as a coenzyme.

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Received December 30th, 1959

Acetyl-coenzyme A in patulin biosynthesis

The hypothesis that acetate condenses directly in biological systems to form aromatic compounds was initiated by COLLIE[1], and was further applied by BIRCH[2] to explain the occurrence of many natural products. Experiments with *Penicillia* spp. fed labeled acetate[3,4] demonstrated that 6-methylsalicylic acid is formed by polymerization of C_2 units; and it was also shown that the antibiotic patulin in turn can result from the enzymically catalyzed molecular rearrangement[4] of 6-methylsalicylate.

In light of recent developments on the biosynthesis of fatty acids and of sterols, it appeared most probable that the activated form of acetic acid utilized in the formation of the foregoing extra-mycelial aromatic and related compounds would by acetyl-SCoA. This report deals with the occurrence of moderately large amounts of coenzyme A in *Penicillium patulum*, and describes the preparation of a cell-free extract which can convert acetyl-SCoA into patulin.

Growth of *P. patulum* was carried out as previously described[5]. When the absorbancy of the fermentation beer at 276 mμ was about 1.0 at a 0.005 dilution, the mold was repeatedly washed with cold water to remove mechanically bound metabolites. The mycelia were then suspended in 0.01 *N* NH_4OH (see ref. 6) (50 ml per mycelial mat from 2.8 l fernbach flasks) and stirred for several hours at 3°. After gravity filtration first through muslin and then through Whatman No. 4 filter paper, the extract was centrifuged in the cold at 5000 × *g*. The resultant clear, slightly yellow solution averaged 0.65 mg protein/ml, measured colorimetrically against a γ-globulin standard by the Folin-Ciocalteau method. This crude preparation was capable of transforming glucose, acetyl-SCoA, or 6-methylsalicylic acid to patulin. All active preparations made in this fashion were found to contain coenzyme A.

Other procedures which were used for making cell-free extracts from *P. patulum* included: grinding in the cold with boat sand, with alumina, with powdered glass, and with combinations of these abrasives in various buffers; and treatment in the cold with abrasives in the Waring blendor. Although considerable protein was liberated by these methods, and although many of these extracts could oxidize glucose, as measured manometrically, they were devoid of patulin or 6-methylsalicylate-synthesizing ability.

The presence of coenzyme A in the mycelium of *P. patulum* was demonstrated by concentration of the crude material, using the methods previously applied[7] to isolation of the coenzyme from yeast. Spectrophotometric assay of the initial hot water extract from mycelial mats indicated that about 50 mg of coenzyme A were present/g (dry wt.) of the mold. The crude coenzyme was precipitated from the aqueous extract by the addition of acetone, and was then reprecipitated from solution as its glutathione complex with Cu_2O. After removal of metal with H_2S, the resultant solution of the partially purified coenzyme was lyophilized to give hygroscopic white needles. A comparison of this material with a commercial sample (Pabst) by spectrophotometry in the u.v. at pH 2.5 and at pH 11 showed that the curves were identical. Analysis by paper chromatography demonstrated that the *P. patulum* coenzyme A was resolved into major spots which had the same R_F values and color reactions[8] as did the authentic yeast coenzyme. Finally, the coenzyme A from *P. patulum* exhibited biological activity in the *Escherichia coli* phosphotransacetylase enzyme system[9]. By this

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Abbreviations: HSCoA, Coenzyme A.

method it was estimated that the best preparation contained about 133 LIPMANN units/mg.

Patulin which was formed in the experiments using cell-free extracts was removed from the reaction mixture by ether extraction. The ether was then washed with 1 % $NaHCO_3$, dried over $MgSO_4$, and passed through a column of Woelm alumina. The eluate was taken to dryness, redissolved in ethanol, and chromatographed serially several times on Whatman No. 1 paper using the butanol–acetic acid–water (4:1:1) solvent system, until constant radioactivity was obtained. Radioactive acetyl-SCoA was synthesized by the method of SIMON AND SHEMIN[10] using [2-^{14}C]acetic anhydride. Labeled 6-methylsalicylic acid was obtained by biosynthesis from an experiment[4] in which *P. patulum* was grown on [2-^{14}C]acetate. [^{14}C]glucose was a uniformly labeled commercial sample.

The results of experiments with these radioactive substrates and the enzyme extract from *P. patulum* are given in Table I. It can be seen from Expts. 1 and 2 that samples of labeled acetyl-SCoA prepared either from the mold or yeast coenzymes were roughly equivalent in their efficiency for incorporation into patulin. Dilution of the specific activity of the resultant patulin in these experiments is undoubtedly due to endogenous formation of the antibiotic from acetyl precursors present in the extract. Acetate itself, added in 60-fold higher radioactive concentration, failed to yield radioactive patulin. This demonstrated both that the necessary acetate-activating components were absent from the extract, and that the mechanism of condensation of labeled acetyl-SCoA did not include prior hydrolysis to free acetate. It had previously been shown[4], with intact mycelia, that glucose is transformed into 6-methylsalicylate and patulin by way of dissimilation to "active acetate". This conclusion is now reinforced by the results of Expts. 4 and 5, where the presence of 10^{-3} M sodium monofluoroacetate in the enzyme system inhibited isotope incorporation from labeled glucose into patulin. In the final experiment it is seen that the enzyme extract catalyzed the molecular rearrangement of 6-methylsalicylate to patulin, and that the product had a specific activity slightly higher than that of its precursor. This result is in accord with previous replacement experiments using mycelial mats, wherein the loss of the non-labeled carboxyl from the alternately tagged aromatic compound to give patulin with four of its seven carbon atoms was

TABLE I

5 ml cell-free extract (3.0 mg protein) were diluted with an equal volume of 0.15 M phosphate buffer, pH 6.8, containing the appropriate radioactive supplement. After incubation at 35° for 4 h, the contents of the flasks were acidified and worked up as indicated in the text.

Expt. No.	*Substrate*	*Specific activity counts/min/μmole*	*Total activity counts/min*	*Specific activity of isolated patulin counts/min/μmole*
1	Acetyl-SCoA (mold)	3,000	30,000	2,200
2	Acetyl-SCoA (Pabst)	1,000	10,000	600
3	Na-[2-^{14}C]Acetate	60,000	600,000	0
4	Glucose	3,000	30,000	1,130
5	Glucose + 10^{-3} M sodium monofluoroacetate	3,000	30,000	0
6	6-methylsalicylic acid	3,750	16,000	3,960

demonstrated. The results of Expt. 6 also indicate that the presence of 6-methylsalicylate in the reaction mixture repressed the endogenous formation of patulin from acetyl-SCoA precursors. This finding, and the fact that only trace amounts of 6-methylsalicylate itself were detected in Expts. 1 to 5 supports the contention that this aromatic compound is not an obligatory precursor of the antibiotic, but that it is in reversible equilibrium with a common acetyl-SCoA-derived open-chain intermediate.

This work was supported by a grant (NSF-G-5902) from the National Science Foundation.

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Received January 4th, 1960

Biochim. Biophys. Acta, 40 (1960) 535–537

An enzymic study on the cellular origin of the DUNNING and the NOVIKOFF hepatomas in the rat

Because of technical difficulties that are difficult to surmount, the literature on the biochemistry of cancer is filled with comparisons of questionable validity. For example, in comparing liver cells and hepatoma cells, any such comparison can be questioned on the basis of whether the hepatoma cells have been derived from parenchymal liver cells*, from bile-duct epithelium, or from still other cells that occur in liver tissue. A number of studies summarized by Potter[3,4] in relation to the "deletion hypothesis" have shown that several enzymes normally found in liver are either missing or present in very small quantities in the Novikoff hepatoma, which has been regarded by some to be derived from parenchymal liver cells (discussed by Novikoff[5]). Several of these enzymes were later reported by Pitot *et al.*[6] to be present in the Dunning hepatoma and these authors questioned the significance of the earlier comparisons of Novikoff hepatoma and liver, while further studies by De Verdier and Potter[7] with the Dunning hepatoma revealed new differences between this tumor and normal and regenerating liver.

Abbreviations: 3′-Me-DAB, 3′-methyl-dimethylaminoazobenzene; dCMP, deoxycytidylic acid; Tris, tris(hydroxymethyl)aminomethane.

* That the parenchymal cells of the liver lobule differ quantitatively in their enzyme content depending on their anatomical location in the lobule has been shown by Shank *et al.*[1] and by Schumacher[2]. Such differences, which are small in comparison with the major fluctuations in enzymic activity shown by the liver as a whole, may reflect the blood flow and oxygen tension in various zones of the liver lobule[2].

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An interesting example giving the appearance of the converse of deletion, *i.e.*, an enzyme not present in liver[8] but present in the NOVIKOFF hepatoma[9], was reported by MALEY AND MALEY, who found dCMP deaminase in the tumor but not in adult rat liver*. Earlier studies on regenerating rat liver by HECHT AND POTTER[10] indicated the use of a pathway from uridylic to thymidylic acid without the necessity for first forming deoxycytidylic acid, and made it unnecessary to assume that deoxycytidylic acid deaminase synthesis was suppressed in normal liver and released or induced in NOVIKOFF hepatoma and regenerating liver.

Prior to the present study DUNNING hepatomas were assayed[11] for dCMP deaminase and appeared to contain none of the enzyme. MALEY AND MALEY[9] reported slight activity of the enzyme in 24–48 h regenerating liver; however, we were not able to reproduce this result consistently. Rats bearing the DUNNING hepatoma were sent to MALEY and MALEY, who confirmed the absence of the deaminase from this tumor[12]. This finding left unexplained the occurrence of considerable amounts of the enzyme in the NOVIKOFF hepatoma[9], a result which we have confirmed. In order to test the hypothesis that the NOVIKOFF hepatoma is derived from a cell type other than that from which the DUNNING hepatoma stems, which could now be argued to be derived from a parenchymal liver cell on the basis of enzymes both present[6] and absent[11], we examined the livers from animals fed a diet[13] containing 0.06 % 3′-Me-DAB** for short periods of time sufficient to produce extensive bile-duct proliferation but no tumors, as shown by PRICE *et al.*[14]. As shown in Table I, these livers contained readily demonstrable amounts of the dCMP deaminase. When the animals were removed from the diet and fed laboratory chow for 2 weeks, the deaminase activity was not

TABLE I

All assays were performed on the S_3 fraction (microsome-free supernatant) prepared from 20 % isotonic KCl homogenates of the respective tissues. The incubation vessels contained 0.5 ml S_3 fraction, 0.2 ml 0.03 *M* dCMP (Calif. Corp. for Biochemical Research) dissolved in 0.2 *M* Tris–HCl buffer, pH 8.0, 0.1 ml 0.1 *M* KF, and 0.2 ml 0.154 *M* KCl[9]. The reaction time was 20 min. The reaction was stopped with 4.0 ml of 1 *N* $HClO_4$. After centrifugation to remove the precipitated protein, the supernatant was neutralized with KOH using phenol red as an indicator. The tubes were left at 0° overnight and the resulting crystals of $KClO_4$ were centrifuged down. The supernatant was decanted onto 6 × 1 cm columns of Dowex 1 in the formate form and the nucleotides and nucleosides separated and analyzed by the method of BRUMM AND POTTER[11]. The enzyme activities are expressed as µmoles deoxyuridylic acid formed/h/g tissue.

Tissue	*dCMP deaminase activity*
Normal liver	0, 0, 0
Embryonic liver (17–20 days)	58, 20
Regenerating liver (48 h)	0, 1, 5
DUNNING L-C18 hepatoma§	0, 0, 0, 0
NOVIKOFF hepatoma§§	70, 59, 66, 41
Liver, 3′-Me-DAB diet – 22 Days	11, 11, 11, 8
Liver, 3′-Me-DAB diet – 36 Days	7, 5, 8, 5
Liver, 3′-Me-DAB diet – 27 days, followed by 15 days Laboratory Chow Diet	0, 0, 0, 0.4

§ Maintained in Fischer strain inbred rats originally supplied by Dr. W. F. DUNNING, U. of Miami, Coral Gables 46, Florida.

§§ Obtained in 1956 from Dr. A. B. NOVIKOFF, Albert Einstein School of Medicine, New York City.

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* We are indebted to MALEY AND MALEY for sending us their manuscript[9] prior to publication.

** These animals were kindly supplied by Drs. J. A. MILLER and E. C. MILLER.

measurable. Histologic sections taken at this time showed an almost complete disappearance of bile-duct proliferation*.

These results suggest the possibility that the Novikoff hepatoma cell is derived from a cell that resembles bile-duct epithelium more than it resembles parenchymal liver cells, while the Dunning hepatoma cell may be more closely related to parenchymal liver cells. It is of interest that despite their marked differences with respect to the deaminase, the two hepatoma strains resemble each other in their weak activity with respect to uracil and thymine deoxyribose transferring enzyme[7] and their inability to catabolize uracil and thymine. The latter results will be reported elsewhere. The biochemical indications for a relationship between the two hepatomas and their possible normal prototypes support a combined biochemical and pathological study undertaken by Pitot, Clark and Farber[6](*cf.* Pitot[15]). Daoust[16] has carried out biochemical and cytological studies on heterogeneous cell populations and perhaps by these or by additional techniques[1] further evidence for the derivation of these hepatomas, as well as others now under study, can be accumulated.

This work was supported in part by a grant (No. C-646) from the National Cancer Institute, National Institutes of Health, U.S. Public Health Service.

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Received December 28th, 1959

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* The sections were obtained through the courtesy of Dr. M. Angevine, Department of Pathology, University of Wisconsin Medical School.

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Biochim. Biophys. Acta, 40 (1960) 537–539

Peroxidase activity found in the ribonucleoprotein particles from pea seedlings and rabbit liver

While studying the RNase and related enzymes in the RNP from pea seedlings, we found that the particles possessed peroxidase activity. Although nucleic acids have been reported to have peroxidase activity[1], the activity in the RNP was too strong to be attributed to the nucleic acids contained in the particles, suggesting that the enzyme peroxidase was present in the RNP. The RNP from rabbit liver also showed peroxidase activity.

RNP from pea seedlings

3-day-old pea seedlings were detached from their cotyledons, cut into small pieces, cooled to 5° and ground in a mechanical mortar with 2 vol. 0.5 *M* sucrose. The ground material was filtered through a cotton cloth and the filtrate was centrifuged at 50,000 × *g* for 30 min in a Spinco preparative centrifuge (with No. 30 rotor.). The supernatant was further centrifuged at 100,000 × *g* for 120 min and the precipitate of RNP (possibly containing small microsomes) was collected, suspended in water, and the suspension centrifuged at 10,000 × *g* for 15 min[2]. $MgCl_2$ was added to the supernatant to a concentration of 0.05 *M* and the precipitate was collected as a RNP preparation[3]. This preparation contained 151 μg RNA-phosphorus and 20 G.U. (guaiacol unit) and 965 B.U. (benzidine unit) of peroxidase/mg N (1 G.U. is defined as the amount of enzyme which produced 1 mg tetraguaiacol at 30° in 15 min[4]. 1 peroxidase unit[5] = 500 G.U.; 1 B.U. is defined as the amount of the enzyme which caused an increase of absorbancy at 600 mμ of 0.1 during the first 5 min of the reaction at 15° in the following reaction mixture: 5 ml 0.1 *M* acetate buffer, pH 5.3, 1 ml 0.05 % benzidine, 1 ml 0.05 % H_2O_2 and enzyme in a total volume of 8 ml.) The pH optimum of the peroxidase was found at 5.3 when guaiacol or benzidine was used as substrate. The enzyme also reacted equally well with other phenols and aromatic amines. It could be solubilized from the RNP by the compounds which combine with metal ions: citrate, phosphate and EDTA (see Table I), suggesting that the enzyme is combined on the particles through metals. Peroxidase is generally present in plant cells in a considerable amount, and in pea seedlings, about 1/3 of the total peroxidase activity was contained in the microsomes and RNP fractions. The pH-5-fraction also had some activity (see Table II). The absorption spectrum of the solubilized enzyme showed a maximum at about 410 mμ. This may be the same as cytochrome b_3 (see ref. 6) which was found in the microsomes from silver-beat petioles and wheat roots, and may also correspond to peroxidase *b* (see ref. 7) which was prepared from broad-bean leaves, because the peroxidase *b* has an absorption spectrum similar to that of the cytochrome *b* group.

RNP from rabbit liver

Rabbit liver was cut into small pieces and was washed with cold physiological saline in order to remove blood. The washed peaces were homogenized and the RNP was fractionated by the same procedure as applied to pea seedlings. The preparation

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Abbreviations: RNA, ribonucleic acid; RNase, ribonuclease; RNP, ribonucleoprotein particles; EDTA, ethylenediaminetetraacetic acid.

TABLE I

SOLUBILIZATION OF THE PEROXIDASE FROM THE RNP

RNP was suspended in the indicated solutions. The pH of these solutions were 6.0 in the case of pea seedling and 5.3 in the case of rabbit liver. The suspensions were centrifuged at 10,000 × *g* for 30 min except in the case of sucrose, when 100,000 × *g* for 120 min was used. The peroxidase activities of both suspensions and supernatants were assayed. In the case of pea seedling, the activity was measured by reading the absorbancy at 470 mμ under the same condition as the B.U. determination except that guaiacol was used as substrate. In the case of rabbit liver, the activity was measured under the same condition as the B.U. determination. The peroxidase activity is expressed in percentage of the rate obtained with the suspension in water.

Treatment	*Pea seedling*		*Rabbit liver*	
	Suspension	*Supernatant*	*Suspension*	*Supernatant*
Water	100	8	100	67
Sucrose, 0.5 *M*			100	20
Acetate buffer, 0.1 *M*	111	22	77	63
EDTA, 0.001 *M* in acetate buffer	111	38	102	63
EDTA, 0.01 *M* in acetate buffer	100	47	97	66
EDTA, 0.1 *M* in acetate buffer	103	45		
Phosphate buffer, 0.1 *M*	106	48		
Citrate–phosphate buffer, 0.1 *M*	97	57	105	59

TABLE II

DISTRIBUTION OF PEROXIDASE IN THE CELL FRACTIONS

Cell debris and large particles (nuclei, mitochondria) were obtained as precipitate by centrifuging the homogenate at 20,000 × *g* for 15 min, and microsomes were separated from the mitochondrial supernatant by centrifuging at 50,000 × *g* for 30 min. Further centrifugation of the supernatant obtained at 100,000 × *g* for 120 min gave the RNP as precipitate. The pH-5 fraction was prepared from the microsomal supernatant by the addition of 0.1 *M* acetic acid to bring its pH to 5.2 and collecting the precipitate by centrifugation. The clear, final supernatant was also used for assay. Peroxidase activities of pea seedling and rabbit liver were assayed by the methods shown in Table I. The peroxidase activity is expressed as percentage of the rate obtained with the whole homogenate in the case of pea seedling and with microsomal supernatant in the case of rabbit liver.

Fractions	*Pea seedling*	*Rabbit liver*
Homogenate	100	
Cell debris and mitochondria	8	
Microsomes	10	
Microsomal supernatant		100
RNP	17	31
pH-5 fraction	18	46
Final supernatant	47	23

was pinkish and contained 21 μg RNA-phosphorus and 8 B.U. of peroxidase activity/mg nitrogen. The peroxidase in the RNP was not lost by washing with 0.5 *M* sucrose, but more than a half of the activity was solubilized by water or acetate and citrate buffers (see Table I). The pH 5-fraction also had peroxidase activity (see Table II). This enzyme from liver has very sharp specificity toward substrates, among which benzidine seems to be the best. The pH optimum was found at the same pH value as that of the pea-seedling enzyme. Although the test with benzidine leaves some room for ambiguity because this substrate can be oxidized even by haemoglobin or haematin, the fact that the peroxidase activity was eliminated by heating while the haematin-catalysed oxidation is resistant to this treatment, was evidence that the peroxidase activity in the preparation was not due to free haematin. This was supported by the fact that the enzyme in the liver RNP was able to oxidize guaiacol. The absorption spectrum of the solubilized enzyme showed a maximum at about 410 mμ; the enzyme may correspond to cytochrome b_5 (see ref. 8, 9) which was found in the microsomes from rabbit liver, and which may also be responsible for the peroxidase activity in bovine liver[10] or rat uterus[11].

Since the amount of RNA in the RNP preparations, especially that from liver, is small, they probably contain other components of the microsomes. This seems to be due to the omission of deoxycholate and buffers which are ordinarily employed for these preparations. Deoxycholate is known not only to solubilize lipids but also to combine metal ions. Therefore, enzymes which are bound on RNP through metal ions are preserved on the RNP prepared without deoxycholate, while elimination of the metal ions by deoxycholate is likely to cause the loss of the enzymes through solubilization in the other preparations.

We have found previously a RNase[12] and related enzymes (3′-nucleotidase and purine nucleoside ribosidase)[13] in the RNP from pea seedlings. These facts suggest that nucleic acid is metabolized in RNP and also that it contains xanthine oxidase or uricase. In fact, the existence of uricase in liver microsomes has already been reported[14]. Both xanthine oxidase and uricase produce H_2O_2, which is necessary for peroxidase activity. When the RNP preparations from pea seedlings and rabbit liver were incubated with uric acid and guaiacol or benzidine at pH 5.3 or 7.5 at 37° for 30 min, coloured products were formed. This showed that H_2O_2 was produced by the uricase reaction. However, the colours were unlike those which appeared when H_2O_2 is added. With guaiacol, the reaction mixture was pink coloured, compared with a brownish yellow when H_2O_2 is added. With benzidine, the reaction mixture was yellow compared to blue. Although some peroxidase[15] can oxidize uric acid, they require the addition of H_2O_2 and the reaction product has no colour.

Thus, the present results seem to allow us to postulate the existence of the peroxidase system linked with nucleic acid metabolism in the RNP. It will be an interesting problem to identify the real substrate of peroxidase in the living cells.

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Received February 8th, 1960

Serotonin receptors

II. Calcium transport by crude and purified receptor

Recent evidence indicates that the probable mechanism of action of serotonin is to bring about the passage of calcium ions through the cell membrane[1]. This passage of Ca^{++} and its reaction with actomyosin + adenosine triphosphate would explain why the hormone causes smooth muscles to contract. The chemical reactions involved are the combination of serotonin with a specific lipid in the cell membrane (the serotonin receptor), followed by the binding of Ca^{++} by the receptor-serotonin compound. The resulting calcium-containing complex is soluble in fat, and is thus able to diffuse through the lipoidal cell membrane. It is in this way that the lipid-insoluble Ca^{++} ion is made to penetrate the lipoidal membrane of the cell. Inside the membrane there seems to be an enzyme which attacks this complex, degrading it, and thereby liberating sertonin + Ca^{++} + degraded receptor[2].

The serotonin receptor has been extracted from smooth muscles and nerves[2], and has been recognized to be a lipid which combines specifically with serotonin, thereby causing it to become soluble in fat solvents. However, the actual transport of calcium in a cell-free system has not yet been demonstrated. The difficulty was that the relatively crude concentrates of the receptor lipid contained considerable calcium combined with the phosphatides so that the actual transport of calcium in response to additions of serotonin could not be clearly established[2]. By the use of radioactive Ca^{++} it has now been possible to show that the lipoidal receptor when combined with serotonin does actually pick up Ca^{++} and make it fat-soluble. When the complex is subsequently decomposed by addition of acid, Ca^{++} is liberated and becomes again water-soluble. Thus, *in vitro* in the absence of cells or cell particles the transport through a lipid phase of Ca^{++} as well as serotonin can be demonstrated. This can be done either with rather crude preparations of the receptor lipid, or with material purified by chromatographic fractionation of the crude extract.

To make these demonstrations, crude receptor was extracted from fresh hog duodenum with chloroform–methanol as described earlier[2]. The extract was concentrated rapidly at low temperature[2], and the resulting residue was suspended in dry chloroform. Anhydrous Na_2SO_4 was added, and the soluble portion was separated, and freed of chloroform under reduced pressure. The lipid mixture so obtained was called crude receptor. This material was purified by a series of adsorptions on columns of silicic acid according to a method about to be published. Purified receptor was thus obtained. It was not, however, a completely pure substance.

The cell-free, *in vitro* system previously used for demonstration of the transport of serotonin through a lipid solvent was used[2]. It consisted of a centrifuge tube containing 2.5 ml benzene, 2.5 ml *n*-butanol, and 4 ml water. The organic layer contained the receptor preparation, and the aqueous phase contained the salts (36 mg NaCl, 1.68 mg KCl, 7.0 mg $NaHCO_3$, 2.0 mg $CaCl_2$, and 100,000 counts (0.04 mg) $^{45}CaCl_2$). One such tube was used as control and a second which contained, in addition, 1 mg serotonin acted as the experimental system. The amount of receptor used in each experiment varied from 4 to 75 units. Additional controls were of course run, from which receptor or serotonin, or both were omitted. After each mixture had been shaken and thoroughly cleared by centrifugation, an aliquot of the organic layer was removed and shaken with 5 ml 0.2 *N* HCl. The aqueous acid extract was washed with benzene to remove the last traces of lipid, evaporated, and the radioactivity counted. Results of a typical experiment are shown in Table I.

It can be seen that the presence of serotonin in this system resulted in the transport through the organic layer of considerable calcium in excess of that which was carried in the absence of the hormone. Note that in the absence of receptor the serotonin caused no transport of calcium. The calcium carried over in the presence of receptor, but in the absence of serotonin, probably was the result of nonspecific impurities in the receptor preparations. Thus, an impurity such as phosphatidic acid would be expected to pick up Ca^{++} from the aqueous phase. The calcium phosphatidate would probably be soluble in the organic layer, and, when this was shaken with aqueous acid, would be converted to phosphatidic acid, and $CaCl_2$. The latter would extract into the final aqueous layer and be counted. Such nonspecific transport of calcium made it essential to show that the addition of serotonin actually brought about a decided increase in the calcium transported in this *in vitro* system. In the living cell, on the other hand, this problem probably does not arise because instead of acid, the cell uses the internal enzyme to decompose the calcium–serotonin–receptor complex. This enzyme presumably has specificity for its substrate, and would not be expected to attack such substrates as calcium phosphatidate.

TABLE I

^{45}Ca TRANSPORTED BY SEROTONIN AND ITS RECEPTOR

Serotonin mg	*Crude receptor units*	*Purified receptor units*	*Total counts/min*
0	0	0	0
1	0	0	0
0	28	0	4420
1	28	0	6330
0	0	5	1630
1	0	5	5740

The nonspecific binding of calcium by impurities in the receptor preparations made it essential to use the large amount of non-radioactive calcium specified in the directions. When the total calcium was reduced to less than 1 mg the differences in counts transported with and without serotonin were diminished. It was necessary to have enough total calcium present to saturate all of the nonspecific binding sites before a satisfactory demonstration of the action of serotonin could be made.

This work was supported in part by a grant from the U.S. Public Health Service.

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Received January 18th, 1960

The formation of spermidine from spermine by serum amine oxidase

The naturally occurring polyamines, spermine and spermidine, are known to be oxidized by an amine oxidase present in beef or sheep serum[1]. The oxidation products obtained by the action of amine oxidase on spermine or spermidine are known to inhibit the growth of *Mycobacterium tuberculosis*[2]. The mechanism of the oxidation of polyamines by serum has not yet been elucidated, and different assumptions exist as to the nature of the growth-inhibiting products. TABOR *et al.*[1], using purified enzymic preparations, suggested that spermine is first converted into spermidine, which is then further oxidized. CARVAJAL AND CARVAJAL[3] proposed a different mechanism, in which spermidine does not appear as an intermediate in spermine oxidation.

During our studies of the inhibitory effect of spermine on the growth of *M. tuberculosis* we were able to show that an amine with an R_F value of 0.13, corresponding to that of spermidine, was formed by the action of serum amine oxidase on spermine. Quantitative determinations of spermidine and spermine were carried out[4] at different time intervals. Results illustrated in Fig. 1 show that after 120-min incubation of spermine with sheep serum, almost all original spermine disappeared. A close correlation was found to exist between the rate of spermidine formation and spermine oxidation, pointing to the possibility that spermine is converted to spermidine during the initial steps of polyamine oxidation by serum amine oxidase. If this is correct, a marked similarity exists between the mechanism of polyamine oxidation by serum amine oxidase and polyamine degradation by bacteria, for both *Pseudomonas aeruginosa*[5] and *Mycobacterium smegmatis*[6] convert spermine into spermidine at the initial step of the oxidation.

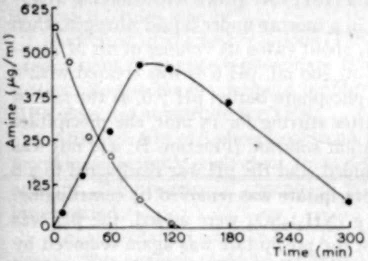

Fig. 1. Formation of spermidine from spermine. 20 µmoles spermine tetrahydrochloride were incubated with 2.4 ml sheep serum and 8.6 ml tris-(hydroxymethyl)aminomethane buffer, pH 6.8. Aliquots were analyzed by quantitative paper chromatography at various time intervals. ○, spermine; ●, spermidine.

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Received January 8th, 1960

Preparation of an active glucose 6-phosphate dehydrogenase from *Neurospora crassa*

During investigations of the biosynthesis of valine and isoleucine in *Neurospora crassa*[1], commercial samples of TPNH prepared by reduction of TPN by $Na_2S_2O_4$ were found to inhibit severely α-keto-β-hydroxy acid reductase. Crude extracts of *N. crassa* contained an active glucose 6-phosphate dehydrogenase and offered a convenient source of this enzyme for the enzymic preparation of TPNH. Purification of the enzyme has yielded fractions of high specific activity with reasonable recoveries.

The assay mixture consisted of enzyme fraction, suitably diluted, 0.1 ml; glucose 6-phosphate, 1.0 μmole; TPN, 0.1 μmole; Tris buffer, pH 7.5, 100 μmoles; all in a total volume of 1.0 ml. The increase in absorbancy was measured at 340 mμ and 25 to 27° in a recording spectrophotometer. A unit of enzyme activity[2] is that amount of enzyme that produces a change of 1.000 absorbancy unit/min under the assay conditions. 6-Phosphogluconate dehydrogenase was measured under the same conditions, but with 6-phosphogluconate replacing glucose 6-phosphate as substrate.

Mycelium from a 60-h culture of *N. crassa* (16117 A) grown with shaking at 37° in a synthetic medium[3] was filtered, ground in a mortar under liquid nitrogen, then homogenized in a Ten Broeck apparatus with about twice its volume of 0.1 *M* phosphate buffer, pH 7.0. This extract (Fraction A, 400 ml, pH 6.8) was treated with a 1.5 % solution of protamine sulfate in 0.1 *M* phosphate buffer, pH 7.0, at the rate of 15 mg protamine sulfate/100 mg protein[4]. After stirring for 15 min, the precipitate was removed by centrifugation. The supernatant solution (Fraction B; 440 ml) was adjusted to pH 7.0, 77.4 g $(NH_4)_2SO_4$ were added, and the pH was readjusted to 7.0 by the addition of NaOH. After 30 min, the precipitate was removed by centrifuging. To the supernatant solution (482 ml), 29.9 g $(NH_4)_2SO_4$ were added, the pH was adjusted to 7.0 with NaOH, and after 30 min the precipitate was again removed by centrifuging. The supernatant solution (488 ml) was finally treated with 30.7 g $(NH_4)_2SO_4$ and the pH again adjusted to 7.0 and the mixture centrifuged after 30 min. The precipitate contains the dehydrogenase and was dissolved in 42.0 ml 0.1 *M* Tris buffer, pH 7.5 (Fraction C). This fraction contained about 62 % of the glucose 6-phosphate dehydrogenase activity originally present in the crude extract and did not dehydrogenate 6-phosphogluconate.

Fraction C (40 ml) was dialyzed for 12–15 h against 6 l 0.005 *M* phosphate buffer, pH 7.5, containing 10^{-4} *M* Mg^{++}. The precipitate formed during dialysis was discarded. The volume of the supernatant solution was adjusted to 80.0 ml with cold distilled H_2O (Fraction C-1). The pH was adjusted to 6.5 by careful addition of 0.1 *N* acetic acid and the precipitated protein removed by centrifuging. The supernatant solution (Fraction C-2) was subjected to fractional adsorption with calcium phosphate gel (OCHOA *et al.*[5]). 10 ml of centrifuged gel (15.4 mg solids/ml) and an absorption time of 15 min, with stirring, were used for each of six steps. Each gel precipitate thus obtained was eluted by stirring for 30 min with 40.0 ml 0.2 *M* phosphate buffer, pH 8.0, and the supernatant solution transferred to the next gel step, in sequence[5]. All operations were carried out at 0–5°. Most of the glucose 6-phosphate dehydrogenase was present

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Abbreviations: TPN, TPNH, oxidized and reduced triphosphopyridine nucleotide; Tris, tris(hydroxymethyl)aminomethane.

TABLE I

SUMMARY OF PURIFICATION PROCEDURE FOR THE GLUCOSE 6-PHOSPHATE AND 6-PHOSPHOGLUCONATE DEHYDROGENASES FROM *N. crassa*

*Fraction**	*Volume ml*	*Protein mg/ml*	*Glucose 6-phosphate dehydrogenase units/mg protein*	*6-phosphogluconate dehydrogenase units/mg/protein*
A	400	12.1	1.95	0.63
B	440	9.0	2.54	0.87
C	42	18.7	7.55	0
C-1	80	6.2	13.3	
C-2	82	4.8	14.2	
C-3	40.5	1.29	35.6	
C-4	40.5	1.10	36.3	
D	42.5	17.3	4.27	1.15
E	46.0	21.7	0	1.15

* See text.

in gel eluates 4 and 5 (Fractions C-3 and C-4, Table I) and the combined recovery in these fractions was 37 %.

Activities of the best fractions (Table I) are higher than those reported for many preparations of glucose 6-phosphate dehydrogenase[2,6,7], and the yield is more satisfactory. No specific requirement for Mg^{++} was found, and under the assay conditions DPN does not substitute for TPN.

Although free of 6-phosphogluconic acid dehydrogenase, the preparation contains hexokinase activity equivalent to 14.5 % of the glucose 6-phosphate dehydrogenase activity. The presence or absence of other closely associated enzymes has not been investigated.

The supernatant solution from Fraction C contained 6-phosphogluconate dehydrogenase. This enzyme could be separated from glucose 6-phosphate dehydrogenase by successive additions of 32.7 g and 114.6 g, respectively, of $(NH_4)_2SO_4$ to precipitate fractions D and E of Table I.

This work was supported in part by a grant (E-1575) from the U.S. Public Health Service to Professor E. E. SNELL. I am indebted to Miss H. SCHENKER for technical assistance and to Dr. SNELL for his interest and encouragement.

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Received January 16th, 1960

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The effect of thymidine upon the incorporation of uracil into *Bacillus cereus*

In a recent publication[1] 6-azauracil was reported to inhibit the transformation of orotic acid into uracil in the organism *Bacillus cereus*. As a result of this lowered uracil production, the synthesis of RNA and of DNA was decreased. By the addition of thymidine to the treated bacteria complete restoration of DNA synthesis could be effected. Under the same conditons RNA synthesis was increased but, nevertheless, remained below the normal level. This latter observation suggested that the thymidine might be converted to a uracil-containing material.

This observation has been further investigated. An experiment was performed in which the incorporation of [2-^{14}C]uracil into the total nucleic acids of the organism was measured in the presence and absence of thymidine. The procedure used was as follows: aliquots (1 ml) of the bacterial suspension in logarithmic growth were taken after varying periods of incubation time and added to 1 ml of cold 10 % trichloroacetic acid, containing a small amount of the unlabelled precursor. The mixture was allowed to stand for 30 min in the cold and then was filtered through a Millipore filter and washed 4 times with 5 % trichloroacetic acid. The filtered material plus the filter paper were placed on copper planchettes, dried and counted. The results shown in Table I indicate that the thymidine considerably decreases the incorporation of the uracil into the total nucleic acid (60 % decrease after 20 min), although total nucleic acid synthesis continues uninhibited.

TABLE I

EFFECT OF THYMIDINE UPON THE INCORPORATION OF [2-^{14}C]URACIL INTO THE NUCLEIC ACIDS OF *B. cereus*

2-^{14}C]Uracil (237 μg/ml and 1.5 μC/ml), 0.7 ml; thymidine (10 mg/ml), 0.6 ml; bacteria, 20 ml.

Incorporation time (min)	*Total counts/min*	
	Labelled uracil	*Labelled uracil + thymidine*
10	37	12
20	114	45
40	378	126
60	776	293

Experiments were performed in order to test the possibility that the thymidine was contaminated with uracil, which would also explain the above results. A uracil-less mutant, *Aerobacter aerogenes* P-12, was kindly provided by Dr. B. MAGASANIK and D. KARIBIAN of the Harvard Medical School. This mutant grows well on either uracil or uridine[2]. A culture of this bacteria was incubated in the presence of thymidine to determine whether it was contaminated with a uracil-containing material. Fig. 1 illustrates the effect on growth of different concentrations of uridine with the effect produced by thymidine. The results indicate that the thymidine does not contain a quantity of uracil (or uracil derivative) which can account for the decreased incorporation in the above experiment. The maximum amount of uracil present in our sample of thymidine cannot exceed 1 μg/300 μg thymidine and in order to produce the dilution

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Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

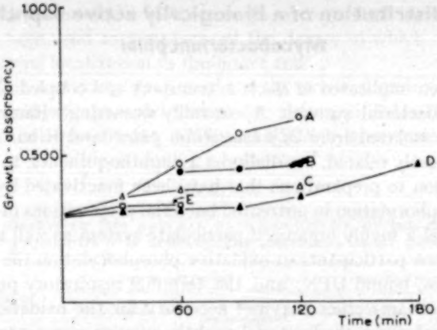

Fig. 1. Growth of *A. aerogenes* P-12 in the presence of uridine and thymidine. Curve A, uridine, 12 μg/ml; B, uridine, 6 μg/ml; C, uridine, 3 μg/ml; E, uridine, 1.5 μg/ml; D, thymidine, 300 μg/ml.

of uracil incorporation recorded in Table I almost 14 μg/ml uracil would have to be added (calculated after 20 min incubation).

There are several possible explanations for the above results the most evident of which is that the thymidine is transformed into a uracil derivative. However, up to the present time no such mechanisms have been observed although reactions in the reverse direction are well known[3]. The work of FRIEDKIN *et al.*[4] on chick embryos and of REICHARD[5] on liver tissue has shown that thymidine apparently is not metabolized to give rise to material which enters RNA. In *E. coli* and *Salmonella typhimurium*[6] similar observations have been made. One further point although it supports this interpretation does not explain all the facts. In the 6-azauracil-treated *B. cereus* thymidine restores only partially the formation of RNA[1]. If thymidine (300 μg) can provide 14 μg uracil this amount would certainly seem ample to restore RNA synthesis completely, unless azauracil affects the conversion of thymidine.

The explanation that thymidine exerts a sparing action on uracil also seems unlikely to account for the available data. In *B. cereus* almost 90 % of the total nucleic acid is in the form of RNA[7]. Another possibility is that the presence of thymidine may affect the penetration of uracil into the cell or the formation of its riboside. Experiments are planned in order to clear up these points.

The author would like to express his thanks to Dr. H. CHANTRENNE for his helpful criticism during the course of this investigation.

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Received December 30th, 1959

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Subcellular distribution of a biologically active naphthoquinone in *Mycobacterium phlei*

Quinones have been implicated in electron transport and coupled phosphorylation in mammalian and bacterial systems. A naturally occurring vitamin K-like napthoquinone has been isolated from *Mycobacterium phlei*[1] and it has been shown, like vitamin K_1 and closely related, 2,3-dialkyl-1,4-naphthoquinones, to restore oxidation and phosphorylation to preparations that have been inactivated by irradiation[2,3].

Coupled phosphorylation in untreated bacterial preparations of *M. phlei* requires the participation of a highly organized particulate system as well as soluble components. Of the known participants in oxidative phosphorylation the particles contain the dehydrogenases, bound DPN, and the terminal respiratory pigments[4], whereas the supernatant contains other enzymes necessary for the oxidation of DPNH and for phosphorylation[5]. Since the bacterial naphthoquinone appears to be an additional component of this system, its distribution and concentration in the subcellular fractions have been determined and compared with those of certain other electron carriers.

M. phlei cells, ATCC 354, were grown, disrupted, and fractionated by methods previously described[5]. The electron carriers analyzed were compared on a molar basis. In order to compare the relative concentrations of the carriers in the particles and the supernatant, the fractions were compared on a dry weight basis.

The total flavins were extracted with 10 % trichloroacetic acid and were measured fluorometrically[6]. The fluorescence of the extract was taken to indicate the concentration of FMN, since free riboflavin has not been found in bacteria[7]. The concentration of FAD was determined by measuring the increase in fluorescence following acid hydrolysis.

To estimate the naphthoquinone, extraction with a mixture of isopropanol–isooctane (3:1) was followed by adsorption of a petroleum ether (30–60°) solution of the material onto Permutit and elution with diethyl ether in petroleum ether. The concentration of the naphthoquinone was determined by the colorimetric procedure of SCHILLING AND DAM[8], with vitamin K_1 as a standard. The values agreed with those obtained from the absorption at 249 mμ, assuming $E_{1\,\text{cm}}^{1\%} = 630$. The identity of the naphthoquinone was confirmed by paper chromatography[1].

In order to compare the concentration of the naphthoquinone with that of terminal electron carriers, cytochrome *b*, chosen for analysis as a representative of the terminal respiratory pigments, was measured by the alkaline pyridine method of PAPPENHEIMER[9]. The difference in absorption at 560 mμ between the oxidized and reduced states was used to calculate the concentration; standard curves were obtained with hematin. Prior treatment of the particulate fraction with rattlesnake venom, a method similar to that of AMBE AND CRANE[10], was necessary to obtain preparations transparent enough for photometric measurements.

It is difficult to describe enzyme systems as particulate or soluble with most disruptive procedures commonly used. However, some degree of reliability can be attained by using as an index of structural integrity a physiological function such as

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Abbreviations: DPN, diphosphopyridine nucleotide; FMN, riboflavin monophosphate; FAD, flavin-adenine dinucleotide.

oxidative phosphorylation for which retention of organized structure is essential. This activity has been used as a measure of the degree to which the system retains the pattern of enzyme localization in the intact cell.

The distribution and concentration of the electron carriers analyzed in preparations capable of oxidative phosphorylation are shown in Table I. The supernatant fraction contained both the flavins, FMN and FAD, in greater concentrations than the particulate fraction. The relatively high concentration of FMN in the supernatant correlates with the observed non-phosphorylative FMN-linked pathway of oxidation catalyzed by this fraction[5].

TABLE I

THE RELATIONSHIP OF THE NATURAL NAPHTHOQUINONE TO THE FLAVINS AND CYTOCHROME *b* IN SUBCELLULAR FRACTIONS OF *M. phlei*

Electron carrier	*Supernatant fraction*		*Particulate fraction*	
	*μmoles/mg dry wt.**	*Relative concentration***	*μmoles/mg dry wt.**	*Relative concentration***
FAD	$2.64 \cdot 10^{-4}$	1.8	$9.85 \cdot 10^{-5}$	0.6
FMN	$5.95 \cdot 10^{-4}$	4	$5.30 \cdot 10^{-5}$	0.35
FAD + FMN	$8.59 \cdot 10^{-4}$	5.2	$1.52 \cdot 10^{-4}$	1
Naphthoquinone	$6.03 \cdot 10^{-4}$	4	$3.52 \cdot 10^{-3}$	23
Cytochrome *b*	0	0	$2.65 \cdot 10^{-4}$	2

* Each value represents the average of at least 4 determinations, except for that of the natural naphthoquinone which was the average of at least 8 determinations.

** The relative concentrations of the electron carriers are expressed as molar ratios, the total concentration of the flavins in the particulate fraction being taken as unity.

Although a small amount of the naphthoquinone was found in the supernatant, the major portion was localized in the particulate fraction. Only one naphthoquinone, with the same R_F value shown by paper chromatography[1], was regularly obtained from both fractions.

As has been observed previously for the terminal respiratory pigments of *M. phlei*[4], only the particles contain cytochrome *b*.

The combined system of the particles and the supernatant, which is capable of coupled phosphorylation, contained the flavins, naphthoquinone and cytochrome *b* in the ratio of 3.4:13.5:1. Thus the quantity of the naturally found naphthoquinone is sufficient to account for function at at least one of the phosphorylative sites in electron transport. There is an apparent excess of the natural napthoquinone. Concentrations of quinones of the same order of magnitude as that of the napthoquinone in *M. phlei* have been found in other bacteria and in mammalian and plant tissues[11]. The fact that vitamin K is necessary for the oxidation of reduced DPN and for the reduction of the terminal respiratory pigments indicates that this coenzyme functions between these two respiratory carriers[12].

It is concluded that the biologically active napthoquinone in *M. phlei* is located in the particles. Its concentration relative to other electron carriers is sufficient to support electron transport and to account for a coenzymic role in oxidative phosphorylation.

ADDENDUM

Quinones from other microorganisms have been examined; however, not with respect to other electron carriers. LESTER AND CRANE[11] have found both a benzoquinone (Q_8) and a naphthoquinone in *Escherichia coli*; the benzoquinone was present in a high concentration only in cells grown aerobically. In the present studies three quinones have been crystallized from *E. coli*: a vitamin K_2-like napthoquinone and two benzoquinones, one with an absorption maximum at 260 mμ and the other at 270–275 mμ. The three quinones were distinguishable by chromatography and were present in cells grown aerobically. Under anaerobic conditions the concentrations of the various quinones were altered. It is difficult to quantitate the quinones because of losses during fractionation and overlapping spectra; however, it appears that under anaerobic conditions the napthoquinone and the 270 benzoquinone are diminished in concentration, while the other benzoquinone is increased. The heterogeneity of these compounds in this facultative anaerobe may provide information concerning the biological role of the various benzo- and naphthoquinones, and the effects of these compounds on oxidative phosphorylation in *E. coli* are being investigated.

This investigation was supported by a research grant (No. E-2674) from the National Microbiological Institute, National Institutes of Health, U.S. Public Health Service.

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Received February 4th, 1960

Biochim. Biophys. Acta, 40 (1960) 550–552

Preparation and properties of glutamic dehydrogenase from human placenta

The oxidation of L-glutamic acid by human placenta has been observed[1] but the properties of the placental enzyme were not studied. The key role of this enzyme in intermediary metabolism and the evidence that its rate is under hormonal control[2] led us to compare the properties of the placental and liver enzymes.

For each enzyme preparation, 2–4 fresh human placentas were trimmed free of cord and membranes, cut into sixths, washed free of adherent blood and immersed in ice-cold 0.9 % NaCl. The tissue was scraped free of gross connective-tissue strands and homogenized in 2 vol. of ice-cold 0.15 *M* KCl for 1 min in a Waring Blendor. The crude homogenate was centrifuged for 10 min at 700 × *g* at 0° in an angle-head centrifuge to remove fibrous tissue and debris. The resulting homogenate was strained through four layers of fine-mesh cheese-cloth and centrifuged at 8000 × *g* for 30 min at 0° to sediment the mitochondrial fraction. An acetone powder of the mitochondria was prepared in the usual way at — 15°, and extracted twice with 10 vol. 0.05 *M* potassium phosphate buffer, pH 7.4, for 30 min at roomtem perature with continuous gentle stirring. The aqueous extract was centrifuged at 8000 × *g* for 10 min at 0° to remove the sediment. 0.5 vol. of Na_2SO_4 saturated at 38° was added to the aqueous extract slowly with continuous gentle stirring, the solution was stirred for an additional 10 min, and the resulting precipitate was removed by centrifugation at 15° for 10 min at 8000 × *g*. 0.33 vol. Na_2SO_4 was added to the supernatant fluid and the fraction precipitating between 33 and 50 % saturation was similarly removed and dissolved in 0.05 *M* potassium phosphate buffer, pH 7.4, of approximately 0.1 the volume of the aqueous extract. The light straw-colored solution could be stored at — 18° for at least one week with less than 5 % loss in activity; thawing and re-freezing did not result in loss of activity.

The assay for glutamic dehydrogenase was similar to that of STRECKER[3] except that the increment in absorbancy between readings made at 30 and 60 sec after addition of the final reactant was taken as a measure of the enzyme activity. The values reported were determined in 0.05 *M* potassium phosphate buffer, pH 7.4. Protein was measured according to LAYNE'S modification of the method of WARBURG AND CHRISTIAN[4]. When the original homogenate was assayed, KCN was added to a final concentration of 10^{-3} *M* and protein was determined by multiplying the nitrogen content by 6.25.

The results of a typical preparation are shown in Table I. Approximately 2000 units of activity in the aqueous extract were obtained from each placenta used in a preparation. Estimation of glutamic dehydrogenase activity of the whole homogenate from four different preparations gave an average specific activity of 0.08; the overall purification of the enzyme ranged between 600-and 1000-fold from the original homogenate and 5-fold from the aqueous extract of the acetone-dried mitochondria. The enzyme preparation was not homogenous in filter-paper electrophoresis in 0.02 *M* barbital buffer, pH 8.6.

TABLE I

PURIFICATION OF GLUTAMIC DEHYDROGENASE FROM HUMAN PLACENTA

Step	*Volume ml*	*Total activity units**	*Total protein mg*	*Specific activity units/mg protein*	*Yield %*
Aqueous extract of acetone powder (4 placentas)	235	9860	811	12.1	100
Na_2SO_4, 33–50% satn.	25	6000	99	60.3	61

* One unit of enzyme is the amount which causes a change in absorbancy of 0.001/min.

Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

TABLE II

MICHAELIS CONSTANTS OF REACTANTS IN GLUTAMATE OXIDATION

Reactant	K_m*	*S.E.*** K_m
Glutamate	$0.44 \cdot 10^{-3}$	$1.55 \cdot 10^{-7}$
DPN	$3.79 \cdot 10^{-5}$	$7.05 \cdot 10^{-8}$
TPN	$7.32 \cdot 10^{-6}$	$1.96 \cdot 10^{-10}$

* Estimated by method of least squares from the usual Lineweaver-Burk transformation in which $Y = 1/v$, $X = 1/[S]$.

** Standard error, calculated according to Snedecor[6] from the least-squares analyses.

Homogenates of liver and kidney from a human fetus of 19 weeks gestational age each contained about 10 times as much activity per mg protein as did term placenta; the placenta of this fetus contained the same amount of activity as term placenta.

Like the liver enzyme[3], human placental glutamic dehydrogenase will accept either DPN or TPN as coenzyme, although the activity with TPN is only about one-third that with equimolar DPN. The pH optimum for the forward reaction (glutamate oxidation) in 0.2 *M* phosphate buffer was 8.5–8.6. This value is identical with that reported by Strecker[3] for the crystalline enzyme from beef liver. The enzyme prepared by Olson and Anfinsen[5] showed a pH optimum at 7.7 under similar conditions. The Michaelis constants (Table II) of the forward reaction agree with those reported for the crystalline enzyme from beef liver. The preparation showed no alcohol dehydrogenase, corticosterone dehydrogenase, or hydrocortisone dehydrogenase activity. Neither L-aspartate nor DL-α-methylglutamate was oxidized by the enzyme under the conditions of the usual assay. Lactate was dehydrogenated at about one-third and L-glutamine at about one-half the rate of glutamate. When these substrates were each added together with glutamate the rates were additive. This suggests that lactic dehydrogenase and glutamine deaminase were present in these preparations rather than that these substances serve as substrates for glutamic dehydrogenase.

The characteristics of L-glutamic acid dehydrogenase of human placenta are very similar to those of the enzyme prepared from beef liver.

These investigations were aided in part by grants from the Charles A. King and Marjorie King Fund and from Grant C 2400, National Institutes of Health, U.S. Public Health Service.

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Received January 15th, 1960

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Crystallization of glucose oxidase from *Penicillium amagasakiense*

Several investigators[1-3] have reported methods of obtaining highly purified preparation of glucose oxidase (notatin) from *Penicillium* cultures and some of the properties of their preparations. A crystalline preparation, however, has not yet been described. Recently, some of the present authors succeeded in obtaining a new strain of Penicillium, *Penicillium amagasakiense*, and found that this strain excreted glucose oxidase into the culture medium in submerged cultures. The culture conditions for the production of glucose oxidase by this *Penicillium* and the microbiological nature of this strain will be published elsewhere. A crude preparation of this enzyme thus obtained is now commercially available from Nagase and Co., Ltd., under the name of "Deoxine". In the present communication the purification of crystalline glucose oxidase from "Deoxine" and some of its properties are described.

100 g "Deoxine" was dissolved in 100 ml 0.1 *M* sodium acetate buffer, pH 4.5, and dialysed against the same buffer overnight at 5°. Insoluble materials were removed by centrifugation and the clear dark-brown supernatant fluid was passed through a column (5 × 25 cm) of Amberlite CG-50, Type II, which had previously been buffered at pH 4.5 with the same buffer. The enzyme and coloured materials were completely adsorbed on the resin. The column was washed with 2 or 3 l of the same buffer until the eluate was colourless. The first part of the washings contained half the total oxidase activity and was clear yellow. It seems likely, however, that this fraction contains a partially modified enzyme, since on further purification of this fraction it is difficult to obtain a crystalline preparation. The native fraction could be eluted with 0.1 *M* sodium acetate buffer, pH 5.0, from a clear yellow band. Other coloured impurities were scarcely eluted by this treatment. To the yellow fraction, ammonium sulfate was added and the fraction precipitating between 60 and 80 % saturation was collected by centrifugation. The precipitate was dissolved in a minimum amount of 0.05 *M* sodium acetate buffer, pH 5.0, and dialyzed overnight against the same buffer at 5°. The dialyzed solution was passed through a column (2 × 70 cm) of Amberlite CG-50, Type II, which had previously been buffered at pH 5.0 with the same buffer, and the enzyme adsorbed on the column was eluted with the same buffer. The main fraction was collected and fractionated with ammonium sulfate as described the above. The precipitate was carefully dissolved in a minimum amount of water and centrifuged. To the deep-yellow coloured supernatant ammonium sulfate was slowly added until a slight turbidity appeared after the salt had been dissolved. The mixture was left at room temperature and crystals began to appear within a day. Crystallization was complete within 2 or 6 days. The crystals appeared as needles as shown in Fig. 1, and were collected by centrifugation and dissolved in a minimum amount of water (272 mg of crystals, yield 13 %). Recrystallization was carried out as described above.

As shown in Fig. 2, the absorption spectrum of crystalline glucose oxidase showed maxima at 278 mμ, 380 mμ, and 460 mμ in the oxidized form and at 273 mμ in the presence of glucose. The peaks at 380 mμ and 460 mμ due to FAD disappeared on reduction of the enzyme with glucose or $Na_2S_2O_4$.

The preparation was completely homogeneous by ultracentrifugal analysis and did not contain catalase.

Abbreviations used: FAD, flavin-adenine dinucleotide; FMN, flavin mononucleotide.

Fig. 1. Crystalline glucose oxidase from *Penicillium amagasakiense* (oxidized form; × 400).

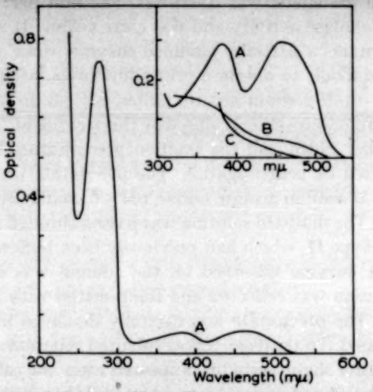

Fig. 2. Absorption spectrum of glucose oxidase. The spectrum was measured with a Cary spectrophotometer Model 14 in a cuvette with a 1-cm light-path, in 0.1 *M* sodium acetate buffer, pH 5.6. Curve A, oxidized form; Curve B, reduced with glucose; Curve C, reduced with $Na_2S_2O_4$.

The sedimentation coefficient ($S_{20,w}$) was 7.93 S and the diffusion coefficient ($D_{20,w}$) was 5.02×10^{-7} cm²/sec based upon determination at four different concentrations of enzyme (0.8–0.2 %) in 0.1 *M* sodium acetate buffer, pH 5.6. The partial specific volume was 0.75. From the above results, the molecular weight was calculated as 154,000. The amount of FAD was found to be 12.8 mμmoles/mg protein by means of the D-amino acid oxidase apo-enzyme test, and the absorbancy at 460 mμ. This result indicates that the equivalent weight per mole of FAD was 78,000. Thus, the enzyme contains 2 moles FAD/mole of enzyme protein. These results are in good agreement of those of CECIL AND OGSTON[4]. The enzyme was stable between pH 3.5

and 7 at 40° and the pH optimum for the glucose-oxidizing activity was at 5.6 at 30°. The activity was measured manometrically in a Warburg apparatus at 30°. The reaction mixture contained 0.2 ml of enzyme solution, 1.2 ml 0.1 M sodium acetate buffer, pH 5.6, and 0.2 ml of catalase solution (prepared by the method of KEILIN AND HARTREE[2]) in the main compartment, 0.2 ml 20 % glucose in the side arm, and 0.2 ml 20 % KOH in the center well. Under these conditions, the turnover number (moles O_2 consumed/mole enzyme/min) was 17,000 (the Q_{O_2} (µl O_2 uptake/mg protein/h) = 148,364 at 30°). The enzyme solution at pH 5.6 could be stored for 2 weeks at 5° without any loss of the activity. The flavin-adenine dinucleotide could be removed from the protein at below pH 2.8 at 0° in the presence of 80 % satd. ammonium sulfate. The activity was regained by the addition of FAD, in proportion to the amount of added FAD. Reactivation was complete on addition of an equal amount of FAD to that liberated. FMN had no effect on the reactivation. The activity was completely inhibited by 10^{-3} M p-chloromercuribenzoate and partially by 10^{-3} M aldehyde reagent such as dimedon, phenylhydrazine, N_2H_4, $NaHSO_3$ and NH_2OH.

The authors wish to express their thanks to Prof. T. ISEMURA and his colleagues of the Institute for Protein Research, University of Osaka, for their technical assistance in measurement of the molecular weight.

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Received February 2nd, 1960

The formation of γ-hydroxy-γ-methylglutamic acid from a common impurity in pyruvic acid

Incubation of a recently purchased preparation of sodium [2-^{14}C]pyruvate with cell-free extracts of *Clostridium propionicum* resulted in the formation of a radioactive dicarboxylic amino acid. Several milligrammes of this product were isolated and identified as γ-hydroxy-γ-methylglutamic acid.

Methods for the cultivation of *C. propionicum* are described elsewhere[1]. Cell-free extracts were prepared by crushing the cells in a Hughes press[2], followed by addition of 2–3 vol. 0.05 M potassium phosphate buffer, pH 7.4, and centrifugation at 20,000 × g in order to remove whole cells and cell-debris.

The amino acid produced on incubation of [2-^{14}C]pyruvate with the extract was isolated by the following procedure. The incubation mixture was treated with 0.1 vol. 10 % $HClO_4$ and centrifuged to remove protein. The supernatant solution was then passed over Dowex 50-H^+ and the material eluted by 1 N NH_4OH was collected and evaporated. An aqueous solution of this material was then put on a 24 × 1.1 cm column of Dowex 1-acetate, X8, 100–200 mesh. The column was washed with 50 ml water and gradient elution was performed by dropping 0.5 N acetic acid into 200 ml water with stirring. At 265 ml a radioactive material appeared in the eluate. At 330 ml the solution in the dropping funnel was changed to 6 N acetic acid and the remainder of the radioactive material was eluted. These fractions were pooled since the radioactive amino acid they contained appeared to be identical on the basis of paper chromatography.

γ-Hydroxy-γ-methylglutamic acid was synthesized chemically by the method of Grobbelaar *et al.*[3]. First the dimer of pyruvic acid, γ-hydroxy-γ-methyl-α-oxoglutaric acid was prepared by the method of De Jong[4] and the dinitrophenylhydrazone derivative was isolated and crystallized, m.p. 173°–177° (uncorr.). (Found: C, 40.34; H, 3.47; N, 15.37. Calc. for $C_{12}H_{12}O_9N_4$: C, 40.45; H, 3.40; N, 15.73.) γ-hydroxy-γ-methylglutamic acid was produced by catalytic reduction of this compound[5]. It co-chromatographed on paper with the radioactive enzymic product in four solvent systems.[*]

Since the amount of radioactive γ-hydroxy-γ-methylglutamic acid formed on incubation of [2-^{14}C]pyruvate with the extract varied with different lots of commercial [2-^{14}C]pyruvate, it appeared likely that the amino acid was derived from a contaminant. Paper chromatography of the dinitrophenylhydrazone of the [2-^{14}C]pyruvate used in these experiments, in the solvent system *n*-butanol–ethanol–0.5 N ammonia (70:10:20) revealed that 80–90 % of the radioactivity migrated with an R_F of 0.20, while only 10–20 % migrated with R_F's of 0.44 and 0.64. The latter two spots are ascribed to the syn and anti isomers of pyruvic acid dinitrophenylhydrazone. The lower spot often appears in chromatograms of pyruvic acid dinitrophenylhydrazone[6].

The synthetic dinitrophenylhydrazone of γ-hydroxy-γ-methyl-α-oxoglutaric acid was compared with the dinitrophenylhydrazone of the contaminant by paper chromatography in two solvent systems: *n*-butanol–ethanol–water (70:10:20), in which the R_F of both was 0.42, and in *n*-butanol–ethanol–0.5 N ammonia (70:10:20), in which the R_F of both was 0.19. Both dinitrophenylhydrazones were subjected to catalytic reduction[5] and the resulting amino acids were chromatographed on paper in 80 % phenol. The R_F of both was 0.16.

The contaminant in [2-^{14}C]pyruvic acid is probably γ-hydroxy-γ-methyl-α-oxoglutaric acid on the basis of this evidence. De Jong observed the formation of this compound from pyruvic acid at room temperature[4].

In view of these results the enzymic formation of γ-hydroxy-γ-methylglutamic acid is thought to occur by transamination. The reactions are:

$$\begin{tikzcd}
\begin{array}{c} 2\,CH_3\cdot{}^{14}CO\cdot COOH \\ \text{Pyruvic acid} \end{array} \arrow[r, "\begin{array}{c}\text{Non-enzymic}\\\text{condensation}\end{array}"] & \begin{array}{c} HOOC\cdot{}^{14}C(OH)(CH_3)\cdot CH_2\cdot{}^{14}CO\cdot COOH \\ \gamma\text{-hydroxy-}\gamma\text{-methyl-}\alpha\text{-oxoglutaric acid} \end{array} \arrow[d, "\text{Transaminase}"] \\
 & \begin{array}{c} HOOC\cdot{}^{14}C(OH)(CH_3)\cdot CH_2\cdot{}^{14}CHNH_2\cdot COOH \\ \gamma\text{-hydroxy-}\gamma\text{-methylglutamic acid} \end{array}
\end{tikzcd}$$

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[*] R_F values of the enzymic and synthetic products were: 0.17 with 80 % phenol, 0.19 with *n*-butanol–acetic acid–water (63:27:10), 0.53 with methanol–water–pyridine (80:20:4) and 0.60 with isopropanol–pyridine–water (1:1:1).

Glutamate was found to be an efficient amino donor in the enzymic step. An alanine-glutamate transaminase has been demonstrated in extracts of this organism[1] and the reaction reported here is probably a non-specific transamination. When purified radioactive pyruvate was used there was no evidence of condensation catalyzed by these extracts. Other aqueous solutions of radioactive pyruvate have been found to be contaminated with γ-hydroxy-γ-methyl-α-oxoglutaric acid and the proportion of contaminant increases with time, even at —20°.

γ-Hydroxy-γ-methylglutamic acid has been found to constitute a large part of the free amino acid pool of the fern *Adiantum pedatum*[3]. It has also been isolated from other plants[7]. LINKO AND VIRTANEN have reported on the synthesis of labelled γ-hydroxy-γ-methylglutamic acid from ^{14}C-labelled pyruvate in whole plants and suggest that this and related amino acids are synthesized from pyruvate by an aldol condensation followed by transamination[8]. FOWDEN AND WEBB[9] have performed similar experiments in a study of the synthesis of γ-methyleneglutamic acid by groundnut plants.

From the results reported here, it is clear that pyruvate should be carefully checked for contamination with γ-hydroxy-γ-methyl-α-oxoglutaric acid in studies concerned with the biosynthesis of possible condensation products of pyruvate. The solvent system, ether–acetic acid–water (13:3:1) provides a simple separation on paper of pyruvic acid, R_F 0.5–0.6, from γ-hydroxy-γ-methyl-α-oxoglutaric acid, R_F 0.1–0.2.

The advice and encouragement of Dr. E. R. STADTMAN during the course of this work and in the preparation of the manuscript is gratefully acknowledged. The author is a Postdoctoral Research Fellow of the National Heart Institute, U.S. Public Health Service.

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Received February 11th, 1960

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Preliminary Notes

Biogenesis of 6-hydroxylated oestrogens in human tissues

Phenolic steroids hydroxylated at carbon atom 6 have recently gained biological interest. Thus Mueller and Rumney[1] incubated oestradiol-17β with mouse liver microsomes in the presence of reduced TPN and obtained evidence for the formation of 6-hydroxyoestradiol-17β and 6-hydroxyoestrone. Breuer, Nocke and Knuppen[2] observed 6-hydroxylation of oestradiol-17β and oestrone on incubation with rat liver slices, and Breuer and Knuppen[3], using the same system, isolated 6-hydroxyoestradiol-17β as a metabolite of oestradiol-17β. Recently Marrian[4] has reported the isolation of a new ketonic Kober chromogen (KC-6B) from pregnancy urine which appears to be identical with a 6-hydroxyoestrone.

There seem to exist two metabolic pathways for the biogenesis of 6-hydroxylated oestrogens in human tissues, one involving the conversion of 6-hydroxylated C_{19}-steroids to the corresponding C_{18}-steroids, and the second which involves the direct hydroxylation of oestradiol-17β. We have examined both possibilities (1) by using Ryan's enzyme preparation of human placenta[5] which converts readily Δ^4-androstene-3,17-dione to oestrone and (2) by carrying out incubations with human fetal liver slices.

Human placentae were prepared as described by Ryan[5]. The 10,000 × *g* supernatant fractions each equivalent to 15 g of tissue were incubated with 10 μmoles ATP, 2.5 μmoles DPN and 200 μg of 6α- or 6β-hydroxy-Δ^4-androstene-3,17-dione at 37° for 60 min in a total volume of 5 ml. The incubation mixtures were extracted with ether–chloroform (3:1, v/v) and the phenolic fractions chromatographed on paper in the formamide–chloroform system. The paper chromatograms were sprayed with Folin and Ciocalteu reagent[6,7]. After incubation of 6α-hydroxy-Δ^4-androstene-3,17-dione a phenolic metabolite (I) was detected which showed the same mobility (14.2 cm/24 h) as authentic 6′α′-hydroxyoestrone prepared by oxidation with chromic acid from 6′α′-hydroxyoestradiol-17β 3-monoacetate 6-monobenzoate[8]. (I) was a Kober chromogen and had the same absorption curve as 6′α′-hydroxyoestrone with the H_2SO_4–H_2O reaction ($\lambda_{\text{max.}}$ 270 mμ, 289 mμ, 462 mμ; $\lambda_{\text{min.}}$ 260 mμ, 275 mμ, 360 mμ). For further identification (I) was sublimed under normal pressure. The crystals obtained had a melting point (261–263°) which was identical with that of 6-dehydrooestrone; the same melting point was observed after sublimation of authentic 6′α′-hydroxyoestrone. The phenolic substance (II) detected after incubation of 6β-hydroxy-Δ^4-androstene-3,17-dione showed a similar mobility (14.6 cm/24 h) as 6′α′-hydroxyoestrone. On rechromatography in the system benzene–light petroleum–methanol–water (33:66:80:20) the mobility of (II) was slightly greater (7.6 cm/24 h) than that of 6′α′-hydroxyoestrone (6.8 cm/24 h). (II) gave a red colour with the Kober reaction. From the data reported here it is concluded that human

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Abbreviations: TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; ATP, adenosine triphosphate.

placenta can convert 6α-hydroxy-Δ^4-androstene-3,17-dione to 6α-hydroxyoestrone and 6β-hydroxy-Δ^4-androstene-3,17-dione to 6β-hydroxyoestrone. It is interesting to note that approx. 4 times as much 6α-hydroxyoestrone was formed as 6β-hydroxyoestrone.

In order to investigate the second metabolic pathway leading to the formation of 6-hydroxylated oestrogens, oestradiol-17β was incubated with human fetal liver. 200 mg of liver slices were shaken with 200 μg of steroid in 5 ml Krebs-phosphate saline at 37° for 60 min. The incubation mixtures were extracted with chloroform–ether (3:1, v/v) and the extracts evaporated. The residues were subjected to paper chromatography in the system formamide–chloroform. The "polar" fractions containing oestriol and possibly 6′α′-hydroxyoestradiol-17β were eluted from the paper and methylated with dimethyl sulfate; the methylated material was then applied to an alumina column (*cf.* ref. 2). Upon elution with 1.4 % ethanol in benzene a Kober chromogen was obtained which showed in the H_2SO_4–H_2O reaction the same absorption curve as the 3-methyl ether of 6′α′-hydroxyoestradiol-17β ($\lambda_{\text{max.}}$ 288 mμ, 438 mμ, 462 mμ; $\lambda_{\text{min.}}$ 260 mμ, 350 mμ, 450 mμ). Further elution with 3.0 % ethanol in benzene yielded the 3-methyl ether of oestriol. On the basis of these results it is concluded that oestradiol-17β is hydroxylated by human fetal liver not only to oestriol—as has already been shown by ENGEL, BAGGETT AND HALLA[9]—but also to 6′α′-hydroxyoestradiol-17β. It seems therefore that, with respect to the metabolism of oestradiol-17β, human fetal liver resembles very closely that of rat liver.

Full experimental details will be published elsewhere. The authors wish to express their gratitude to Professor M. EHRENSTEIN, University of Pennsylvania, Philadelphia, for a generous gift of 6α- and 6β-hydroxy-Δ^4-androstene-3,17-dione, and to Professor C. DJERASSI, Wayne State University, Detroit, for a sample of 6-dehydrooestrone. This work was supported by the Deutsche Forschungsgemeinschaft.

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Received March 23rd, 1960

Rapid incorporation of [^{14}C]-glucose into a glucan-peptide complex of the rat diaphragm *in vitro*

By incubation of rat diaphragm in a medium containing [^{14}C]glucose it has been shown that ^{14}C is distributed into glycogen and CO_2 (ref. 1), as well as into lactic acid, hexose phosphates and an oligosaccharide fraction[2]. In the present work rapid distribution of ^{14}C from [^{14}C]glucose in the medium into two new compounds of rat diaphragm have been observed. One of these compounds has been identified as a glucan–peptide complex.

The experiments were performed as follows: 4 hemidiaphragms were preincubated for 15 min at 20° in phosphate–saline medium without addition of glucose, in order to remove free extracellular glucose. The diaphragms were then transferred to a vessel containing 2 ml phosphate-saline medium. The gas phase was oxygen; the incubation technique has been described earlier[3]. To each vessel was added 10 μC of [^{14}C]glucose (uniformly labelled) which was diluted to a specific activity of 3.6 μC/mg. Incubation was performed for 3 min at 30°. The diaphragms were then rapidly transferred to a stainless-steel mortar, frozen in liquid air, and ground. Extraction was performed with 0.6 N $HClO_4$, followed by centrigufation and neutralization of the supernatant. The supernatant was subjected to paper chromatography in different solvents and to electrophoresis on paper at different pH values. The radioactive zones were detected and quantitated with a paper-strip scanner, Radio Papierchromatograph Frieseke & Hoepfner, GMBH.

Fig. 1 represents the result of a typical radio paper chromatogram with butanol–acetic acid as the solvent. In addition to radioactive glucose and lactic acid two other radioactive compounds appear on the chromatogram, compound A and compound B. Compound A has been characterized as a complex between glucose and a peptide by the following observations. Compound A behaves as a single component in 5 different chromatographic systems. These include butanol–acetic acid–water (R_F, 0.03), phenol–water (R_F, 0.04), acetone–acetic acid–water (R_F, 0.70), picric acid–tert. butanol–water (R_F, 0.42), and butanol–ethanol–NH_3–water (R_F, 0.03). Also by electrophoresis at pH's 4.2 and 10.7, compound A migrates as a single component, negatively charged.

In further experiments compound A was purified on a preparative scale by the following procedure: (a) paper electrophoresis at pH 4.7, identification by autoradiography, and elution from the paper; (b) dialysis of the compound for 18 h against distilled water; compound A was found in the dialysate, while compound B was retained within the dialysis bag; (c) after concentration, the solution containing compound A was subjected to paper chromatography with butanol–acetic acid as the solvent. By this procedure compound A was separated from other radioactive material and from other compounds which gave ultraviolet absorption or positive staining reactions for phosphorus or ninhydrin. In particular, complete separation from hexose phosphates was obtained.

Compound A has been characterized as follows: (1) it possesses a net negative charge at pH's 4.2 and 10.7; (2) it gives a positive ninhydrin reaction; (3) it gives a positive reaction for carbohydrate on paper by spraying with anilin hydrogen phthalate[4], in solution a positive reaction for hexoses was obtained with the anthrone method[5]; (4) it is stable in 1 N HCl for 3 h at 110°. By hydrolysis in 2 N HCl for 8 h at 110° compound A is split, and two main components are formed which chromatographically are identified as glucose and levulinic acid. Compound A is destroyed by treatment with 0.2 N NaOH for 4 h at 110°. (5) After complete hydrolysis of compound A in 6 N HCl for 16 h at 110° ten amino acids were detected by two-dimensional paper chromatography. (6) The presence of radioactive glucose in compound A was demonstrated chromatographically on a hydrolysed sample of the compound. No indication of other hexoses nor of glucosamine has been obtained. (7) Compound A gave no characteristic ultraviolet absorbtion at wavelengths between 240 and 300 mμ. No phosphorus was detected in a sample of compound A after wet ashing.

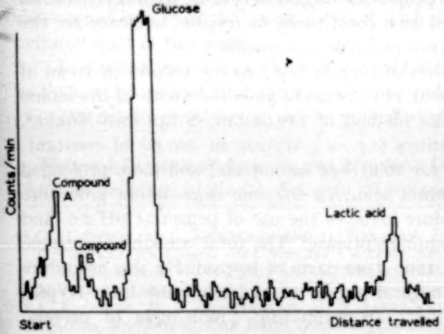

Fig. 1. Radio paper chromatogram of rat-diaphragm-muscle extract. Incubation of rat diaphragm in saline-phosphate medium containing 10 μC [^{14}C]glucose. Incubation time 3 min at 30°. The diaphragms were extracted in 0.6 N $HClO_4$. Paper chromatography of the supernatant in butanol–acetic acid–water (4:1:5).

According to these results compound A has been identified as a glucan–peptide or a glucan–polypeptide complex. The possible significance of this complex as a carrier in the "membrane transport" of glucose is under investigation. Recently similar hexose–protein complexes have been isolated from the membrane of yeast cells[6]. In this connection it seems of considerable interest that insulin added *in vitro* accelerates the incorporation of [^{14}C]glucose into compound A of rat diaphragm. An insulin effect of the magnitude of 200 % has been observed.

A complete report of this work will soon be published.

This work has been supported by grants from the Rockefeller Foundation, from Nordisk Insulinfond and from The Norwegian Council for Science and the Humanities.

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Received March 7th, 1960

Occurrence of hydroxylysine in trypsin

The reaction of trypsin with diisopropyl phosphofluoridate results in the rapid incorporation into the protein molecule of one diisopropylphosphoryl group which has been shown to be bound to a serine residue[1]. A second diisopropylphosphoryl group is incorporated into the trypsin molecule at a much slower rate than the first[2]. In an investigation of the nature of this second phosphorylation site a phosphorus-containing peptide was obtained from partial hydrolysates of trypsin treated with diisopropyl phosphofluoridate, in which a basic amino acid other than lysine, histidine, or arginine was detected. Because its properties suggested it to be hydroxylysine, an amino acid not previously recognized as a constituent of trypsin, evidence for the presence of hydroxylysine in the trypsin molecule was sought.

Acid hydrolysis of small quantities of trypsin (*e.g.*, 20 mg trypsin in 10 ml of constant-boiling HCl in a sealed tube at 110° for 20 h) gave fair yields of the amino acid in question, as measured by the method of SPACKMAN, STEIN AND MOORE[3]. However, hydrolysis of larger quantities (*e.g.*, 2 g trypsin in 200 ml of constant-boiling HCl in sealed tubes at 110° for 20 h) was incomplete, and more prolonged heating led to severe losses of the amino acid. An enzymic degradation procedure was therefore developed. This procedure involves the use of pepsin at pH 2.0, and subtilisin[4], cotazym[5], and purified aminopeptidase[6]. The total amount of enzyme used for degradation was 1 part per 1000–1500 parts of trypsin. By this procedure it has been possible to obtain the natural isomer of hydroxylysine from trypsin hydrolysates in stoichiometric amounts approximating 1 mole/mole of protein. A control consisting of the hydrolyzing enzymes without trypsin yielded less than 1 % as much hydroxylysine as was obtained from trypsin.

The identity of the amino acid with hydroxylysine has been based on the following observations:

(1) The amino acid appears on a column chromatogram[3] in the same position as does the natural isomer of hydroxylysine.

(2) Short-term heating of the amino acid in acid causes the appearance of twin peaks on column chromatograms which are characteristic of hydroxylysine and allohydroxylysine. When this material is chromatographed on a column[3] together with an authentic sample of a mixture of these stereoisomers of hydroxylysine, the two pairs of peaks super-impose exactly.

(3) A sample of the amino acid separated by the chromatographic procedure of HAMILTON[7] from the remaining amino acids shows the same electrophoretic mobility as an authentic sample of hydroxylysine.

(4) The amino gives the characteristic fluorescence in ultraviolet light when sprayed with a specific reagent for hydroxyamino compounds in which the hydroxyl and amino groups are adjacent[8].

(5) The amino acid behaves as hydroxylysine in two-dimensional paper chromatography[9, 10].

The enzymic hydrolysis enables us to obtain hydroxylysine in apparently very good yields. Another interesting feature of this procedure is that it also makes it possible to determine the tryptophan content of the protein. In three different experiments 3.9–4.0 moles of tryptophan and 0.75, 0.90 and 1.05 moles of hydroxylysine/mole of trypsin were found.

Acid hydrolysates of a variety of bovine trypsin preparations have been investigated. Twice-recrystallized trypsin from Worthington Biochemicals and from Mann Chemicals, a five-times recrystallized preparation from Worthington Biochemicals, a three-times recrystallized preparation made in our laboratory and a three-times recrystallized preparation of diisopropylphosphoryl-trypsin were included in these studies. The presence of hydroxylysine, demonstrated in hydrolysates of all of these preparations, suggests that this amino acid is a constituent of the trypsin molecule.

In a previous amino acid analysis of trypsin reported from this laboratory[11] the hydroxylysine was undetected, apparently because the basic amino acids were determined by use of the 15-cm column[2] for basic amino acids instead of the 50-cm column[3] used in this work.

In one analysis of an acid hydrolysate of bovine chymotrypsin a total of 0.6 mole of hydroxylysine and allohydroxylysine per mole of protein was found.

The details of this work will be published elsewhere.

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Received March 28th, 1960

Biochim. Biophys. Acta, 40 (1960) 564–565

A nucleotide enzyme complex associated with fowl leukemia virus

The virus particles that can be isolated from blood plasma in the case of fowl myeloblastic leukemia possess a high magnesium-stimulated ATPase activity[1–3]. ADP has been claimed to act as a competitive inhibitor in the hydrolysis of ATP[4].

A closer study of the viral ATPase activity reveals properties, some of which indicate a complex of phosphorylating enzymes. If activated by Mg^{++}, two pH optima can be distinguished (pH 7.0 and 8.5). Besides Mg^{++}, also other metal ions activate

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Abbreviations: NTP, ATP, UTP, CTP and GTP for the 5′-triphosphates; NDP, ADP, UDP, CDP and GDP for the 5′-diphosphates; NMP, AMP, UMP, CMP and GMP for the 5′-monophosphates of nucleosides, adenosine, uridine, cytosine and guanosine; P_i, inorganic orthophosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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the enzyme complex in the proportions: Mg^{++} 1.0, Cd^{++} 0.5, Fe^{++} 0.5, Zn^{++} 0.25 and Cu^{++} 0.25.

The enzyme complex splits inorganic orthophosphate from nucleoside diphosphates at pH 7.0 in the sequence of activities: ADP < UDP < CDP < GDP, and at pH 8.5 UDP < ADP = CDP < GDP. The quantitative analysis of these reactions shows that at least two events take place: $NDP \rightleftharpoons NMP + P_i$ (ref. 5, 6); and $n\ NDP \rightleftharpoons (NMP)n + n\ P_i$ (ref. 8). To these reactions can be added the ATPase activity: $ATP \rightarrow ADP + P_i$, when ATP is used as the substrate.

Thus, in addition to a high triphosphatase activity, a polynucleotide-phosphorylase (polyase) activity is present, which reaction runs most efficiently if GDP is used as the substrate (GDP ≫ CDP > UDP > ADP, see Table I).

TABLE I

Enzyme reactions with fowl leukemia virus isolated from blood plasma[2,14]. Viral enzyme concentrations, 2.0 and 8.0 µg N/ml reaction mixture[15]. Incubation medium: 0.25 *M* sucrose and 0.04 *M* Tris-buffer. P_i estimation according to ref. 16. The analyses of the polyase, nucleoside diphosphatase and myokinase activities were made in principle as described by Beers[17]. Chromatography of the reaction mixtures was performed on Whatman paper with the isobutyric acid–NH_3–EDTA mixture as solvent. The spots were eluted with 0.1 *N* NH_4OH for spectrophotometric determinations.

Substrate	*µmoles Pi/mg N/30 min Substrate, 2.5 mM; Mg⁺⁺, 3.5 mM; sucrose-Tris; 37°*		*(NMP)n as µmoles NMP/mgN in 30 min*	*µmoles Pi/mg N in 30 min*
	pH 7.0	*pH 8.5*	*Substrate, 6.1 mM; Mg⁺⁺, 1.5 mM; Tris, pH 8.5; 37°*	
ADP	36.6	40.6 –	4.61	38.0
UDP	40.0	33.3	7.32	29.4
CDP	48.4	41.3	11.95	40.0
GDP	58.9	53.9	65.0	58.0
ATP	364.0	408.0	> ADP	445.0

The synthetisizing polyase activities are associated with a diphosphatase and possibly also a myokinase-like activity according to the terminology of Gibson *et al.*[7]. This conclusion is drawn from the decrease of AMP during the later part of the incubation (> 30 min, ATP as substrate, see Fig. 1), as well as from the simultaneous decrease of P_i in the same period. The sequence of activities when different nucleoside diphosphates are incubated is similar to that of the polyase: GDP > CDP > ADP > UDP.

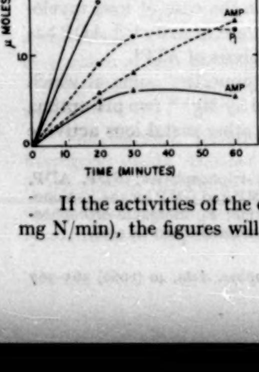

Fig. 1. Viral enzyme complex incubated with 2.5 m*M* ATP (——) or 4.0 m*M* ADP (– – –). Incubation medium (I.M.): 0.25 *M* sucrose; 0.04 *M* Tris, pH 8.5; 1.5 m*M* Mg^{++}; 37°. ADP ○, AMP △, P_i ●.

If the activities of the enzyme complex are expressed in specific activities (units/mg N/min), the figures will roughly correspond to the specific activities measured in purified enzymes from other sources, *e.g.* the adenylate kinase, nucleoside diphosphatase from liver[6,7]. The six to ten times higher activity of the GMP phosphorylase in comparison with the other polyase activities is noteworthy from the point of view of specificity and heterogeneity[8-10] of the polyase enzymes and also of the tumor polynucleotides[11].

Finally, it should be mentioned that ATPase activity with characteristics of the electron-transport chain is also present. Perhaps the viral enzyme complex represents a reminiscence of those parts in the host cell which have participated in the virus synthesis[12]. The view[13] "that viruses do not act only as cell and tissue destroyers but as parts of normal animal cells" conforms with this hypothesis.

This work was supported by a grant from the Swedish Cancer Society.

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Received March 25th, 1960

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Biochim. Biophys. Acta, 40 (1960) 565–567

Interference with adenine and histidine metabolism of microorganisms by aminotriazole

3-Amino-1,2,4-triazole (aminotriazole), a heterocyclic nitrogen compound used extensively in agriculture as a weed killer, bears a close structural relationship to 4-aminoimidazole. 4-Aminoimidazole is recognized as an intermediate in purine degradation in *Clostridium cylindrosporum*[1] and 4-aminoimidazole ribonucleotide is an intermediate in purine biosynthesis[2]. From these considerations, it might be anticipated that aminotriazole could interfere with reactions of purine metabolism and indeed RABINOWITZ AND PRICER have demonstrated[1] that aminotriazole blocks the enzymic conversion of 4-aminoimidazole to formiminoglycine in extracts of *Cl. cylindrosporum*. In the present investigation, it was found that aminotriazole (10 μmoles/4 ml) was inhibitory for growth of *Escherichia coli* and a yeast, *Torula cremoris*. Adenine partially overcame the inhibition in *E. coli* (Table I). In more detailed studies with *E. coli*, not reported here, adenosine, adenylic acid, and guanine were less effective than adenine in relieving the inhibition whereas hypoxanthine and xanthine were totally without effect. The specificity of adenine in counteracting the action of aminotriazole suggested that histidine also be included in this study in view of the demonstrated involvement of adenosine triphosphate in histidine biosynthesis in microorganisms[3]. The data of Table I further illustrate that in *E. coli* the addition of histidine alone had no effect in reversing aminotriazole inhibition but when tested in the presence of adenine, the inhibition was completely reversed even at the 100 μmole level of aminotriazole. With *T. cremoris*, adenine was incapable of counteracting the effect of aminotriazole, but the single addition of histidine completely nullified the toxicity of aminotriazole. One interpretation of the data is that aminotriazole blocks the biosynthesis of histidine or possibly its utilization in both organisms but that in *E. coli* it may also block a step in the biogenesis of adenine. These findings may have a bearing on the mechanism of action of amino-

TABLE I

EFFECT OF ADENINE AND HISTIDINE ON TOXICITY OF AMINOTRIAZOLE FOR GROWTH OF MICROORGANISMS

(Additions to a final volume of 4 ml medium)

Inhibitor 3-amino-1,2,4-triazole μmoles	*Metabolite Growth (absorbancy at 660 mμ) after 24 h*				
	None	*adenine*		*histidine*	
		1 μmole	*5 μmoles*	*1.5 μmoles*	*1.5 μmoles + 1 μmole adenine*
		Escherichia coli			
0	0.69	0.73	0.75	0.74	0.75
10	0.03	0.58	0.60	0.01	0.77
20	0.01	0.43	0.39	0.00	0.75
40	0.00	0.19	0.15	0.00	0.69
80		0.00	0.00		0.71
100		0.00	0.00		0.71
		Torula cremoris			
0	1.0	1.0	1.0	1.0	
10	0.15	0.19	0.26	1.0	
20	0.08	0.09	0.12	1.0	
60	0.0	0.04	0.05	0.95	
100	0.0	0.0	0.0	0.93	

Escherichia coli, Crooks strain, was cultured in a 4-ml volume in 18 × 150 mm test tubes in the synthetic medium of DAVIS AND MINGIOLI[4]; a 24-h culture grown up in this medium was diluted 1:10 and a drop of this suspension added per assay tube as inoculum; cultures were slanted to favor aerobic conditions and incubated for 24 h at 30°. The technique for culturing *Torula cremoris* A.T.C.C. No. 2512 has been previously described[5] and assays were carried out in a final volume of 4 ml in 50-ml Erlenmeyer flasks and placed on a reciprocal shaker for 24 h at 25°. The yeast cultures were then diluted to 10 ml with water before making absorbancy measurements.

triazole as a weed killer, but also suggest that this compound may be useful in biochemical or clinical studies relative to purine metabolism.

Aminotriazole was generously supplied by Dr. T. H. JUKES, Agricultural Division, American Cyanamid Co., Stamford, Connecticut. This work was supported in part by a grant from the National Vitamin Foundation Inc., New York, N.Y.

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Received February 29th, 1960

Biochim. Biophys. Acta, 40 (1960) 567–569

Formation of tryptophol in the disulfiram-treated rat

In man, the major deamination product of norepinephrine is 3-methoxy-4-hydroxymandelic acid[1]. In the white rat, however, a major metabolite of this catecholamine has been shown to be 3-methoxy-4-hydroxyphenylglycol[2]. These metabolites are presumably the oxidation and reduction products of a common intermediate, 3-methoxy-4-hydroxyphenylglycol aldehyde, which is formed *in vitro* by incubation of normetanephrine with purified monoamine oxidase[3]. The existence of this reduction product suggests that other biogenic amines also may be metabolized to alcohols. This possibility was considered for tryptamine which *in vivo* is largely metabolized to indole-3-acetic acid following oxidative deamination[4].

The postulated metabolite of tryptamine, tryptophol (indolylethyl alcohol), was synthesized by reduction of indole-3-acetic acid with $LiAlH_4$ (ref. 5) (this procedure appears useful for the preparation of alcohols from acidic metabolites of many biologically important amines). The leaflets obtained on crystallization from ether–petroleum ether melted at 59° and the picrate melted over a range of 93–96° (*cf.* ref. 6: m.p., 59°; m.p. picrate, 94–96°).

After chromatography in isopropanol–7 N NH_3 (4:1), and in benzene–propionic acid–water (2:2:1), the papers were sprayed with 0.5 % *p*-dimethylaminolbenzaldehyde in 50 % methanol–2 N HCl. A dark blue spot quickly appeared which had the R_F of 0.9 in either solvent. In contrast with tryptophol, indole-3-acetic acid yields maximal color formation with this spray in 20–40 min.

Each of 3 adult white rats was injected intraperitoneally with 10 mg tryptamine and their urines collected in dilute acid over a 24-h period. The pooled urines were adjusted to pH 1 with HCl, heated to 100° for 10 min and then extracted with 4 vol. ether. The ether solution was concentrated by evaporation and a portion of the extract transferred to Whatman No. 1 filter paper. After development in isopropanol–NH_3, the sprayed chromatogram revealed only a single blue spot which corresponded in R_F, color and rate of color development with indole-3-acetic acid.

The absence of tryptophol suggested that the presumed intermediate, indole-3-

acetaldehyde, was completely oxidized. In an attempt to inhibit the oxidation of the aldehyde and perhaps allow formation of the alcohol to take place, an inhibitor of acetaldehyde oxidation, disulfiram[*], was administered to the rats. This drug, which is used in the management of alcoholism, inhibits liver aldehyde dehydrogenase and many other oxidative enzymes[7]. Since disulfiram is insoluble in water, a suspension of 50 mg disulfiram/ml propylene glycol was prepared and 1 ml of this mixture injected intraperitoneally into each of 3 rats daily for 3 days. About 18 h after the last injection, each rat was injected with 10 mg tryptamine and their urines collected and processed as before. The visualized chromatogram showed indole-3-acetic acid and an additional compound. This compound had the same R_F, blue color and prompt color formation as authentic tryptophol. These results are illustrated in a chromatogram represented by Fig. 1. Chromatography in 2 dimensions further confirmed the identity of this compound with tryptophol and also revealed the presence of another acidic metabolite of tryptamine which migrates with indole-3-acetic acid in isopropanol–NH_3 but less rapidly in benzene–propionic acid–water.

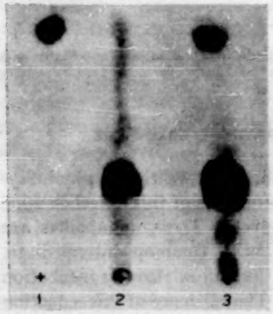

Fig. 1. Paper chromatogram showing presence of tryptophol in an extract from the urines of disulfiram-treated rats injected with tryptamine. The chromatogram was developed by the ascending method in isopropanol–7 N NH_3 and then sprayed with *p*-dimethyl-aminobenzaldehyde reagent. 1, synthetic tryptophol; 2, urine extract from untreated rats showing only indole-3-acetic acid; 3, urine extract from disulfiram-treated rats showing both indole-3-acetic acid and tryptophol.

The simultaneous occurrence of tryptophol and indole-3-acetic acid in the urines of disulfiram-treated rats suggests that they arise from a common aldehyde in accordance with the scheme[2] advanced for the analogous metabolites of normetanephrine. The oxidation of the aldehyde intermediate is apparently inhibited by disulfiram which suggests that this agent inhibits the oxidation of β-substituted acetaldehydes as well as acetaldehyde.

This study was supported by a grant (M-1434) from the U.S. Public Health Service.

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Received March 14th, 1960

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[*] Disulfiram (tetraethylthiuram disulfide, Antabuse) was a gift from Dr. Robitscher of Ayerst Laboratories.